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PRINCIPAL INVESTIGATOR: Stephen J. Doxsey, Ph.D.

CONTRACTING ORGANIZATION: University of Massachusetts Medical Center
                                            Worcester, Massachusetts 01655

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Stephen J. Doxsey, Ph.D.

E-Mail: stephen.doxsey@umassmed.edu

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14. ABSTRACT

Centrosomes are tiny structures critical for organizing mitotic spindles and segregating chromosomes during mitosis. We (and another group) were the first to discover that centrosomes were abnormal in nearly all malignant human tumors. Our recent observations show that centrosome defects and genomic instability can be artificially induced by elevating the levels of the centrosome protein pericentrin and that pericentrin was elevated in prostate carcinomas. Based on these observations, we proposed that centrosome dysfunction may be a critical factor in prostate cancer progression that could explain most phenotypic changes that occur during progression of prostate carcinomas. The specific aims of the original proposal were designed to test several features of this model. 1. Are centrosome defects present in early prostate cancer and can they predict aggressive disease? 2. Do pericentrin's oncogenic features result from the interaction with PKA,PKB/Akt and/or PKC? 3. Can prostate tumor cells be arrested in the cell by overexpression of a pericentrin domain that blocks the cell cycle? We anticipate that this work will lead to new prognostic markers and novel cancer-specific therapeutic targets for aggressive prostate cancer, the form that is most critical in terms of diagnosis, treatment and health care expenditure.

15. SUBJECT TERMS
Centrosomes, Microtubules, Mitotic Spindle, Centrosome Instability, Aneuploidy, Checkpoint Control, Cytokinesis, Prognostics, Theraputics, PIN Lesions, Pericentrin, Centrolin, PKA, PKB, PKC, Cancer Progression, Gene Silencing.

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INTRODUCTION

Centrosomes are essential organelles that control many cellular functions (Doxsey et al, 2005a; 2005b, appendix). They are critical for organizing mitotic spindles and segregating chromosomes during mitosis. They also control cell shape and cell polarity, which are fundamental properties of epithelial gland organization. We (and another group) were the first to discover that centrosomes are structurally and numerically abnormal in nearly all malignant human prostate tumors (Pihan et al, 1998). This observation has important implications for cancer progression since it suggests that centrosome defects might contribute to cytologic anaplasia and genomic instability that so often accompany advanced prostate cancer. Support for this idea came from our recent observations that centrosome defects, cytologic anaplasia and genomic instability could be artificially induced in nontumor cells by elevating the levels of a single centrosome protein called pericentrin and that pericentrin was elevated in many prostate carcinomas and pre-invasive lesions (Pihan et al, 2001; Pihan et al, 2003). Based on these observations, and the knowledge that clinically aggressive prostate carcinoma (high Gleason grade) exhibits significant anaplasia, epithelial de-differentiation and genomic instability, we proposed an innovative hypothesis: that centrosome dysfunction may be a critical factor in prostate cancer progression. We believe that progressive centrosome dysfunction is the first biologic factor identified that can fully explain most of the phenotypic changes characteristic of prostate carcinomas during their progression from clinically indolent forms (majority) to clinically aggressive forms (minority). The specific aims of the original proposal were designed to test several features of this model. 1. Are centrosome defects present in early prostate cancer and can they predict aggressive disease? 2. Do pericentrin's oncogenic features result from the interaction with protein kinases? 3. Can prostate tumor cells be arrested in the cell cycle by overexpression of a domain of pericentrin that drives cells out of cycle? We anticipate that this work will lead to new and powerful prognostic markers as well as novel cancer-specific therapeutic targets for clinically aggressive prostate cancer, the form of prostate carcinoma that is clinically most critical in terms of diagnosis, treatment and health care expenditure. Along these lines, we have licensed two of our cancer patents and entered into a sponsored research agreement with Cytyc, Inc who is interested in developing clinical assays for testing centrosome defects as a prognostic marker of aggressive cervical disease (see Reportable outcomes on Cytyc and related newspaper articles).

REPORT BODY

In this annual report, we summarize progress toward the goals in this proposal. To achieve these goals, we optimized high-resolution imaging methods and quantitative assays to monitor centrosome defects and cell cycle defects. During the course of these studies, we also made some unexpected discoveries. We identified several additional pericentrin interacting proteins that induce aneuploidy when disrupted. Our recent results are summarized below, in abstracts attached at the end of this section and in appendicies at the end of this report.

Pericentrin, aneuploidy and PKA/B/C. We originally showed that elevation of the levels of the centrosome protein pericentrin induced centrosome defects, genetic instability and anaplasia in human prostate cells (Pihan et a, 1998). We now show that the mechanism by which pericentrin accomplishes this is complex. It is in part through misregulation of PKC as originally proposed (Chen et al, 2004, appendix) with contributions from PKA and PKB/Akt. In fact, we found that pericentrin anchored PKA, PKB and PKC at midbodies and that pericentrin depletion by RNA interference mislocalized the kinases from this site. Importantly, we show that disruption of the kinases themselves by RNA interference or dominant-negative expression, caused cytokinesis failure and that disruption of pericentrin's interaction with PKA and PKC give the same phenotype. In all cases, cells fail to divide after replicating both DNA and centrosomes creating polyploid with amplified centrosomes. The extra centrosomes can, in turn, form multipolar spindles that missegregate chromosomes and lead to profound genetic instability. This demonstrates a role for all three kinases in aneuploidy. This work is currently being submitted for publication (see abstract).

Pericentrin, aneuploidy and other proteins. Pericentrin's ability to induce aneuploidy is not so simple however, as several other proteins contribute to aneuploidy. First, we found that disruption of pericentrin's interaction with gamma tubulin also perturbed spindle function leading to aneuploidy. This work was published recently (Zimmerman et a, 2004, appendix). Second, we found that pericentrin interacted with the nucleosome remodeling deacetylase complex (Sillibourne et a, in revision, see abstract and appendix). We discovered that disruption of this interaction also induced tumor like defects in normal cells similar to those seen in cells with abrogated pericentrin. Third, we found that the human tumor suppressor survivin induces its tumorigenic phenotype (eg. aneuploidy) by altering microtubule dynamics during mitosis, cytokinesis and in interphase cells (Rosa et al, 2006, appendix). Forth, we showed that disruption of members of the NFkB pathway leads to mitotic defects and aneuploidy (Rottenberg et al, submitted, see abstract and appendix). Fifth, pericentrin is cleaved by MT1-MMP membrane metalloproteinase and overexpression of MT1-MMP leads to spindle defects, aneuploidy and tumor like features (Golubkov et al, 2005a, 2005b, appendix). Mutation of the pericentrin domain containining the MT1 cleavage site prevents aneuploidy and tumorigenesis (Golubkov et al, 2006). Finally, we found that pericentrin interacts with intraflagellar transport proteins and a calcium channel (polycystin 2) and affects primary cilia growth (Jurczyk et al, 2004, appendix). Collectively, these results suggest that the function of pericentrin is complex and is likely modulated through many of its unique interacting proteins (PKA, PKB, PKC, NuRD, NEMO, centriolin (below), IFTs, polycystin 2, MT1-MMP).

Pericentrin and the prevention of aneuploidy by a centrosome damage checkpoint. During the course of these studies, we found that depletion or overexpression of pericentrin arrests normal diploid cells in the G1 stage of the cell cycle (Mikule et al, 2007, appendix). This arrest is dependent on the tumor suppressor p53, as well as other cell cycle and signaling molecules including p21, p38 and cyclinA/Cdk2 complexes. We also show that heating cells causes pericentrin to disassemble and induce G1 arrest and that reassembly of pericentrin onto centrosomes allows cells to begin cycling again. This has important implications for hyperthermia treatment of cancers as it would likely arrest normal cells but may allow tumor cells to continue dividing and possibly inducing cell death in the process. We show that the mechanism of G1 arrest by pericentrin is induction of defects in centrosome duplication, which is initiated at the G1/S transition. To our surprise, this effect is not specific for pericentrin as depletion of 13 other proteins induces centrosome defects and G1 arrest. In conclusion, we believe that we have identified a novel checkpoint that monitors completion of centrosome duplication and controls entry into the next cell cycle. In this regard the checkpoint is much like the DNA damage checkpoint that arrests in G1 when DNA is not appropriately replicated or if DNA is damaged. This checkpoint would prevent duplication of aberrant centrosomes in normal cells but would allow this defective process to continue in tumor cells lacking p53 or cell cycle control elements. This was published recently in Nat Cell Biol and highlighted in Nat Cell Biol as "checking centrosomes" (Mikule et al, 2007, appendix). In this study we also show that pericentrin and other centrosome proteins are involved in centrosome duplication, growth of primary cilia and centrosome structure.

Pericentrin, centriolin, aneuploidy and cytokinesis. As part of Aim 2, we investigated the relationship of pericentrin and another of its many of its many interacting proteins, centriolin. Pericentrin and centriolin co-localize at the midbody, their localization is co-dependent and their depletion results in cytokinesis failure and polyploidy/aneuploidy. Some of these results were recently published in Cell (Gromley et al, 2005, appendix). Both proteins induce cytokinetic defects by disrupting secretory vesicle fusion at the midbody, a novel observation for cytokinesis and aneuploid generation in human cells. Centriolin and apparently pericentrin, anchor protein complexes required for membrane secretion (exocyst, SNAREs) and mediate the massive membrane secretion that induces abscission, the process by which the cell breaks to form two independent daughter cells. This shows that failure of secretion in the midbody provides a novel pathway to produce aneuploid cells. We have discussed these and other findings two recent reviews covering these topics (Doxsey et al, 2005a, 2005b, appendix, Pihan and Doxsey, 2003). These and other publications can be found in the Reportable Outcomes section.

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Progress on Aim 1 has not yet been possible due to the dissolution of our collaboration with Walter Reed Hospital, which was precipitated after the 911 disaster. We had hoped to obtain these relatively rare samples from Dr. Albrecth Reith at The Norwegian Radiumhospital, Institute for Cancer Research, Norway. However, a fraud scandal resulted in a shut down of all outside collaborations (see Reports/investigation, appendix). We hope to resume work on this important and interesting aim in the near future. Progress made in areas in addition to those originally proposed may compensate somewhat for the lack of progress on Aim 1.

Abstracts related to this proposal

PKA and AKT are anchored at midbodies by pericentrin and control cytokinesis and maintain genetic stability. Purohit A, Mikule K, Doxsey S. In preparation. Pericentrin is a centrosome protein involved in spindle organization and maintenance of genetic stability. Here we show that pericentrin is localized to the midbody during cytokinesis and it anchors three kinases at this site, PKA, PKB and PKC. We show that depletion of pericentrin mislocalizes these three kinases from the midbody and inhibits the final stages of cytokinesis. This results in aneupoid/polyploid cells with extra centrosomes that subsquently organize abnormal spindles and missegregate chromosomes leading to further aneuploidy. Remarkably, disruption of each kinase on its own induces cytokinesis failure. These results reveal new roles for these three three kinases in cell separation and the maintenance of genetic fidelity.

Sillibourne J, Sinah M, Delaval B, Doxsey S. Chromatin remodeling proteins anchor pericentrin at centrosomes and regulate centrosome integrity. Mol Biol Cell, in revision.
Pericentrin is an integral centrosomal component that anchors regulatory and structural molecules at centrosomes. In a yeast two-hybrid screen with pericentrin we identified chromodomain helicase DNA-binding protein 4 (CHD4/Mi2b). CHD4 is part of the multiprotein nucleosome remodeling deacetylase (NuRD) complex. We show that many NuRD components interacted with pericentrin following immunoprecipitation of endogenous proteins and they localized to centrosomes and midbodies. Over-expression of the pericentrin-binding domain of CHD4 or another family member (CHD3) dissociated pericentrin from centrosomes. Depletion of CHD3, but not CHD4, by RNA interference dissociated pericentrin, g-tubulin and other centrosome components from centrosomes. Microtubule nucleation/organization, cell morphology and nuclear centration were disrupted in CHD3-depleted cells. Spindles were disorganized, the majority showing reduced microtubule number and a prometaphase-like configuration. Time-lapse imaging revealed mitotic failure prior to chromosome segregation and cytokinesis failure. We conclude that pericentrin forms a complex(es) with CHD3 and CHD4 and that a distinct CHD3-pericentrin complex is required for centrosomal anchoring of pericentrin/g-tubulin and for centrosome integrity.

A role for IKK in mitosis. Sven Rottenberg[1,3], Keith Mikule[2,3], Rebekka Schwab[1,4], Volker T. Heussler[1,5], Nico Angliker[1], Hana Blazkova[1], Jacqueline Schmuckli-Maurer[1], Paula C. Fernandez[1,6]. (The first two authors contributed equally to this work. Submitted. The IKB kinase (IKK) complex is best known as a central regulator of NF-kB-dependent transcription in response to external environmental signals[1]. Evidence is emerging that IKK translocation and intracellular localisation may determine the specificity of the activated pathway and its biological outcome. Here we show that both pharmacologic inhibition of IKK and depletion of the IKK regulatory subunit NEMO by RNA interference (RNAi) affect several mitotic cell cycle transitions including entry into mitosis, progression from metaphase to anaphase and cytokinesis. Regulation of mitotic progression by IKK is independent of de novo transcription. IKK localises to the centrosome throughout the cell cycle while the activated form can be detected at spindle poles in mitosis and at the midbody during cytokinesis. Stimulation of the classical NF-kB pathway does not result in the accumulation of activated IKK at the centrosome. In summary, we demonstrate a previously unsuspected role for NEMO/IKK in mitotic progression.

KEY RESEARCH ACCOMPLISHMENTS

AIM 1.
Initiated new collaborations to analyze PIN lesions with Dr. Reith (Institute for Cancer Research, Norway). Fraud scandal led to termination of all collaborations (see appendix). This aim remains to be addressed.

AIM. 2.
Disruption of the interaction of pericentrin with PKC induces aneuploidy (Chen et al, 2004).
PKB/Akt disruption leads to aneuploidy (in preparation)
PKA disruption leads to aneuploidy (in preparation)
All three kinases are anchored at the midbody by pericentrin (in preparation)
All three kinases are required for cytokinesis (in preparation)
The pericentrin binding protein, centriolin is involved in cytokinesis and membrane trafficking (Gromley et al, 2005)
Pericentrin is involved in cytokinesis and aneuploidy apparently through membrane trafficking like centriolin
   (S. Mirabelle and S. Doxsey, unpublished observations)
Remnants of cell division mark older daughter cell from the younger daughter cell--implications for aging (Gromley et al, 2005)
Pericenrin and other centrosome proteins are involved in centrosome duplication, primary cilia assembly and centrosome integrity (Mikule et al, 2007; Jurczyk et al, 2004)

AIM 3.
Disruption of pericentrin and gamma tubulin complex proteins induces spindle defects and aneuploidy (Zimmerman et al, 2004)
Pericentrin anchors gamma tubulin at the centrosome during mitosis not interphase (Zimmerman et al, 2004)
Pericentrin interacts with CHD3/4 of the NuRD complex, a transcriptional repressor (Sillibourne et al, in revision)
The NuRD complex anchors pericentrin to the centrosome, the opposite result of that anticipated (Sillibourne et al, in revision)
Disruption of the pericentrin-NuRD interaction or of CHD3/4 alone causes spindle defects and aneuploidy (Sillibourne et al, in revision)
Pericentrin depletion induces cell cycle arrest (Mikule et al, 2007)
Pericentrin is cleaved by MT1-MMP membrane metalloproteinase (Goubkov et al, 2005)
Overexpression of MT1-MMP leads to spindle defects, aneuploidy and tumor like features (Goubkov et al, 2005).
Mutation of the MT1 cleavage site on pericentrin prevents aneuploidy and tumorigenesis (Goubkov et al, 2006).
The human tumor suppressor survivin alters microtubule dynamics during mitosis, cytokinesis and in interphase cells (Rosa et al, 2006).
Changes in microtubule dynamics leads to spindle defects, cytokinesis failure and aneuploidy (Rosa et al, 2006).
Cell cycle arrest is not pericentrin-specific--13 other centrosome proteins induce G1 arrest (Mikule et al, 2007)
Cell cycle arrest is dependent on the tumor suppressor p53, as well as p21, p38 and cyclin A/Cdk2 (Mikule et al, 2007)
This work has important implications for cell cycle progression in the absence of p53 or other regulatory
   molecules implicated in cancer (Mikule et al, 2007)
In fact, suppression of the G1 arrest by abrogation of any of the above molecules leads to spindle or cytokinesis failure and aneuploidy (Mikule et al, 2007)

REPORTABLE OUTCOMES.

♦ **Manuscripts supported by D.O.D. during the entire funding period are represented by asterisks (*) and are found in the References section after the Conclusions section:**

♦ **Presentations by S. Doxsey on prostate cancer-related topics in 2003-2006.**

2003:
03/2003, ***Plenary Lecturer, Conference, Union of the Swiss Societies for Experimental Biology,*** "Emerging roles of centrosomes", Davos, Switzerland.
03/2003, Clark University, Department of Biology, Worcester, MA**.**
04/2003, University of Pittsburgh, Department of Biological Sciences, Pittsburgh, PA.
05/2003, Wistar Institute, University of Pennsylvania, Philadelphia, PA.
06/2003, ***Conference, FASEB Summer Research Conference,*** Nuclear Structure and Cancer. "Centrosome genes in checkpoint control, cell cycle progression and cytokinesis", Saxtons River, VT.
07/2003, ***Conference, American Society for Cancer Research****,* Washington, D.C.*,* RNAi: Opportunities and Challenges in Cancer Research, "Centrosome genes involved in cell cycle progression and genetic instability", Washington, D.C.,
07/2003, ***Conference, Marc-A-Thon***, "Emerging Roles of Centrosomes", Boston, MA,**.**
09/2003, ***Conference of the Chilean Society of Biochemistry and Molecular Biology***, Mechanisms of Cell Division, Concepcion, Chile.
12/2003, ***Conference, American Society for Cell Biology****,* Organelle Maintenance and Inheritance, Minisymposium, "siRNA-mediated centrosome damage activates a G1 checkpoint", San Francisco, CA.

2004:
01/2004, Harvard University Department of Molecular Cell Biology, Cambridge, MA.
01/2004, ***Conference on Aneuploidy and Cancer***, SICR, Oakland California.
01/2004, UMass Medical School, Cancer Center, Worcester, MA.
01/2004, Duke University, Department of Cell and Molecular Biology, Durham, NC.
02/2004, Pfizer, Inc, Groton Research Laboratories, Groton, CT.
03/2004, National Cancer Institute, Microtubule and Mitosis Group of the Screening Technologies Branch, Washington, D.C.
03/2004, Tufts University School of Medicine, Department of Anatomy and Cell Biology, Boston, MA.
03/2004, UMass Medical School, Department of Cell Biology, Worcester, MA.
06/2004, ***Conference on Cytokinesis (ASCB Summer Meeting),*** "Centrioin-anchoring of exocyst and SNARE complexes at the midbody is required for localized secretion and abscission during cytokinesis", Burlington, VT
06/2004, ***Conference on Genomic Integrity in Cancer, General Motors Research Foundation***, "Centrosome-mediated mechanisms of genetic instability", Washington, D.C.
09/2004, ***Keynote Speaker, Conference on Highlights in Basic and Translational Cancer Research,*** "Emerging roles of centrosomes", Rotterdam, The Netherlands,
09/2004, ***6th International Workshop on Chromosome Segregation and Aneuploidy***, "Centrosome genes involved in cytokinesis and chromosome missegregation", Tuscany, Italy.
08/2004, ***Asia-Pacific Conference of Tumor Biology and Medicine, "***Centrosomes and tumorigenesis", Xi'an, China**.**
10/2004, University of New Mexico, Department Molecular Genetics & Microbiology, Albuquerque, NM.

2005:
01/05, University of Connecticut Health Sciences, Dept. Medicine, Framingham, CT

02/05, Assumption College, Worcester, MA
02/05, *Live Cell Imaging Conference*, Hong Kong, China. *Imaging centrosomes in cancer.*
10/2005, "UMass Conference, *UMass: Innovation Partner for the Medical Device Industry, (for members of MassMEDIC).* Prognostic potential of centrosome defects in cancer. Newton, MA
10/2005, Harvard Institutes of Medicine, Dept. Molecular and Developmental Genetics, Boston, MA
04/05. Cytyc Corporation, Inc, Marlboro, MA
06/05, Burnham Institute, La Jolla, CA
06/05, *FASEB meeting on nuclear structure and cancer. Centrosomes and aneuploidy*
06/05, *Gordon Research Conference, Centrosomes and cytokinesis*
07/05, Burnham Institute, La Jolla, CA
09/05, *Co-organizer, Centrosome and spindle poles Conference,* Heidelberg, Germany
11/05, *American Society of Nephrology,* Philadelphia, PA
12/05, *American Society for Cell Biology,* 3 posters
10/05, "UMass Conference, *UMass: Innovation Partner for the Medical Device Industry, (for members of MassMEDIC).* Prognostic potential of centrosome defects in cancer. Newton, MA
10/05, Harvard Institutes of Medicine, Dept. Molecular and Developmental Genetics, Boston, MA
11/05, UMass Research Retreat, Woods Hole, MA

2006:
02/06, Max Planck Institute of Molecular Cell Biology and Genetics, Dresden, Germany
03/06, University of Texas South Western, Dallas, TX
04/06, The Norwegian Radium Hospital, Institute for Cancer Research, Oslo, Norway
04/06, Oregon Health Science Center, Portland, OR
04/06, University of Oklahoma Health Science Center, Oklahoma City, OK
05/06, Curie Institute, Research Section, Paris, France
06/06, Columbia University, New York, NY
06/06, Astra Zeneca, Waltham, MA
07/06, Swiss Federal Institute of Technology, Zurich, Switzerland
11/06, Wadsworth Center, Albany New York
12/06, *American Society for Cell Biology,* 4 posters

♦ **Patents.** Licensed two patents to Cytyc, Inc. for cancer detection and prognosis: *Cancer Detection by Centrosome Abnormality* (#5,972,626) and *Cancer Prognosis by Centrosome Detection*

♦ **Development of permanent cell lines:**
Developed cell lines expressing GFP-centrin2, GFP-gamma tubulin, GFP-GapCenA, RFP-signal peptide

♦ **Development of method for quantitation of centrosome fluorescence intensity.** Developed method for simple quantitation of centrosomal immunofluorescence (see Mikule et al, 2007).

♦ **Institutional and departmental support based on research supported by this award.** The P.I. (SJD) was given departmental and institutional funds ($240,000) to purchase a spinning disc confocal microscope to continue work on cytokinesis and aneuploidy related to this project.
♦ **Institutional and departmental support based on research supported by this award.** Based on work related to this project on cytokinesis and aneuploidy the P.I. (SJD) was chosen to apply for a highly competitive W.M. Keck Foundation proposal. The application was chosen to move to Phase II. Site visit is planned for March 6th, 2007.

♦ **Engaged in sponsored research agreement with Cytyc, Inc. (2005-2007)** to determine if centrosome defects are a prognostic indicator of aggressive cervical cancer.

♦ **Engaged in sponsored research with AstraZeneca Inc. (2006-2008)** to test anti-cancer drugs in the clinic for effects on mitosis and cytokinesis.

♦ **Engaged in sponsored research with AstraZeneca Inc. (2007-?)** to test a panel of drugs for those that affect asymmetric midbody inheritance (Gromley et al, 2007).

♦ **Other Relevant Items (News articles):**
Media 1: Article discussing our paper showing that centriolin depletion induces cytokinesis failure and aneuploidy--appeared in the Telegram & Gazette (Worcester, 10/10/05)
Media 2: Article in Boston Globe on same study as above (10/10/05.
Media 3: Article in Focus (UMass publication) on above (11/05)

CONCLUSION

We believe that our work on centrosome dysfunction will have a significant impact on our understanding of prostate cancer progression, the etiology of prostate cancer and treatment of the more aggressive and devastating forms of this disease. Insights gained from this approach should yield novel information on cellular processes, structures (centrosomes) and molecules (pericentrin, centriolin, Akt) that have the potential to serve as therapeutic targets and prognostic indicators of malignant disease. Support for this idea comes from our recent studies. The discovery that the putative prostate cancer oncogene pericentrin induces aneuploidy by cytokinesis failure and inhibition of secretion, and that pericentrin can induce cell cycle arrest in cells with the tumor suppressor p53 but not in p53-deficient cells, supports this model. The ability to block the cell cycle in prostate cells by depletion of any of 14 centrosome proteins identifies several novel targets for prostate cancer therapeutics. This may provide a mechanism to halt tumor production. In conclusion, we believe this research could provide novel and more discriminating tools for prostate cancer prognosis and treatment.

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appointed by

Rikshospitalet – Radiumhospitalet MC and

the University of Oslo January 18, 2006

Submitted June 30, 2006

To Rikshospitalet – Radiumhospitalet MC and the University of Oslo

The investigation committee appointed on 18 January 2006 hereby submits its report.

The report is unanimous.

Oslo, June 30, 2006

Anders Ekbom
Chair

Gro E M Helgesen   Tore Lunde

Aage Tverdal   Stein Emil Vollset

Sigmund Simonsen
Secretary

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1 Summary

1.1 Appointment

On 18 January 2006, the Rikshospitalet–Radiumhospitalet Medical Center and the University of Oslo (UiO) jointly appointed a special commission to conduct an independent investigation in accordance with detailed terms of reference.

The background for the investigation was that a researcher employed by these institutions, Jon Sudbø, had admitted fabricating the raw data used for a scientific article published in the renowned medical journal *The Lancet* in October 2005.

1.2 The investigation

Early in the investigation it became clear that the entire body of Sudbø's scientific work from 1993-2006 (at least 38 publications) would have to be scrutinized, and that the coauthors (60 altogether) would in reality also have to be subject to investigation. All the authors received a letter requesting them to submit a voluntary written statement, which they all did. Moreover, information was gathered from relevant institutions and other relevant partners. Special mention should be made of the findings from the thorough investigations made by the Cancer Registry of Norway. The Commission also met with individuals and representatives of institutions, including Jon Sudbø. Furthermore, the Commission has obtained documents and other information from several other sources. Available data lists, etc., and published research results have been correlated and compared. Accordingly, the Commission was generally able to judge whether, and the extent to which, the underlying data on which the publications are based are genuine. As its main principle, the Commission has found it appropriate to apply a standard of proof based on a *qualified* preponderance of probability as a condition for accepting a particular fact as grounds for the report.

1.3 Findings

Jon Sudbø began his PhD project in 1993 under the supervision of Albrecht Reith. The PhD project consists of two separate parts. One part involves theoretical and applied works on tissue architecture in cancerous tumors and normal tissue. The Commission has not found indications of research flaws related to these works. As reflected in his subsequent research, most of his PhD project involved characterizing the early stages of oral cancer. The research question was whether and, if so, to what extent, different types of classifications of white patches in the oral cavity were indicative of a high risk for developing oral cancer. The doctoral dissertation and related publications give an affirmative response to this question, asserting that a

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classification based on DNA content can with great accuracy predict the subsequent development of cancer.

First published in the highly respected *New England Journal of Medicine* in 2001, this sensational finding was based on DNA analyses of 150 patients with leukoplakia (i.e. 'white patches' that may be early stages of oral cancer) in the oral cavity. In 2004, a second article was published in the *New England Journal of Medicine*, based on further investigations of the same 150 patients. Based on their own investigations and those made by the Cancer Registry of Norway, the Commission's point of departure is that there are serious problems associated with this crucial patient material. For instance, the same patient appears several times. As far as the Commission can determine, the material consists of 141 different patients at the most, since several patients are represented by several tissue samples that collectively add up to 150. Further, the Commission has found that 69 of the 141 patients included in the study should have been excluded because they had been diagnosed with oral cancer before or at the same time as the leukoplakia was diagnosed. For these patients, it was not possible to study the future development of cancer, since they already had cancer. This error alone is so serious that the results and the conclusions are invalid. The Commission has also uncovered several other inconsistencies. For example, the age distribution in the data files is not consistent with the underlying patient material. Further, the Commission has noted that the reported 150 DNA analyses are to some extent repetitions of data from a far smaller number of patients. The reporting on how DNA analyses and the classification of leukoplakia were conducted (by several observers) is also incorrect and misleading.

Consequently, the Commission has determined that the data underlying parts of the PhD project, as well as several other publications, are not sufficiently consistent with the actual facts the Commission has found it reasonable to take into account. The internal affairs investigation conducted by the Cancer Registry of Norway has arrived at the same conclusion.

The Commission is of the opinion that the errors and defects that have been exposed are too numerous, too great and too obvious to be attributed to random errors, incompetence or the like; and that the raw data therefore appear to have been fabricated, manipulated and adapted to the desired findings.

The consequence of this is that the doctoral dissertation and three related original articles must be retracted. In addition, subsequent publications must be retracted where they are based on the same raw material, as most of them are. On the same grounds, the Commission also questions one other original article. Further, the Commission has questioned an original article published in the *Journal of Clinical Oncology* 2005, *inter*

alia in the light of circumstances partially acknowledged by Sudbø. The most recent original article published in *The Lancet* in 2005 has been retracted, since it is, in its entirety, based on fabricated raw data. Jon Sudbø has admitted this.

This means that the bulk of Jon Sudbø's scientific publications are invalid due to the fabrication and manipulation of the underlying data material.

1.4   Criticism, possible explanations and preventive measures

The exposed fabrication and manipulation of research data justify criticism against Jon Sudbø. The comments that Sudbø has made to the Commission in a meeting and after having read two draft reports with attached documentation, have not given the Commission reason to make any major changes in the preliminary conclusions drawn during the investigation.

In compliance with the terms of reference, the Commission has posed the question of how such – in retrospect – obvious and gross acts could have been perpetuated over such a long period of time in collaboration with so many well-qualified coauthors/ scientists and research institutions.

The Commission points out that there will invariably be certain possibilities for a dishonest researcher to dupe and deceive others. Another factor is that Jon Sudbø has operated relatively independently both as a doctoral candidate and later as a researcher. He has always maintained full and sole control of the underlying data. In that connection, the Commission has found reason to criticize his supervisor for a lack of due diligence and academic supervision during Sudbø's fellowship. This case has also revealed what appears to be a systemic failure at the Norwegian Radium Hospital with respect to a lack of supervision, training and control procedures. Another circumstance is that there has been no formal permission or approval whatsoever of the project on the part of external bodies, nor has anyone taken it upon themselves to arrange for or check this. In this context, it has been noted that the institutions that contributed patient material have not required verification of the necessary permits, e.g. dispensation from mandatory confidentiality.

The Commission has not found indications that others, including some of the coauthors, have been involved in the fabrication and manipulation of research data or by other means been party to scientific misconduct. However, in good conscience and based on cost/benefit considerations, the Commission has not perceived its task as being to investigate less serious types of deviations from the norm. The co-authors can generally be divided into two groups: 1) suppliers (subcontractors), and 2) higher level guarantors

(senior researchers), who to little or no degree contributed to or had knowledge of the underlying data material. Most communication has taken place through Jon Sudbø. Thus the co-authors have had little opportunity, as well as little reason, to check the underlying data and each other's contributions. Such a division of labour is not uncommon for medical publications that must necessarily be based on cooperation between researchers with rather dissimilar professional backgrounds and tasks, and thus require that they trust each other.

On the other hand, the Commission has pointed out certain factors to which several people should have reacted, be they co-authors, supervisors, superiors, opponents, colleagues or others. Since there have been a number of less serious mistakes on the part of several people that must be viewed in context (collective and cumulative mistakes), the Commission has found reason to view this as systemic failure, where the responsibility rests with the institutions.

In light of this, the Commission has recommended that the institutions take more responsibility for raising awareness and instructing their researchers about the rules that apply, and that they engage in at least a minimum of verification and control, taking appropriate account of academic freedom.

The Commission has not perceived its task as being to expose specific damaging effects. This will probably be a topic for a subsequent investigation by the Norwegian Board of Health. Notwithstanding, the Commission has noted that colleagues, researchers, clinicians and individual patients have probably used Sudbø's research results, and it is therefore reasonable to assume that some of them have been affected. The serious implications of this must have been obvious to Jon Sudbø right from the start.

1.5   The Commission's Report – an overview

Chapter 2 of the investigative report presents the conditions of the Commission's appointment, the terms of reference and methods of working. The chapter discusses the investigative principle adopted, mode of information retrieval, the principle of contradiction, standards of proof, the relationship to disclosure, and thresholds for criticism.

In Chapter 3, the Commission has found reason to outline the ethical and legal framework that applies to medical and health research. Here, the Commission provides a general review of the rules of authorship and supervision, etc.

Chapter 4 reviews the facts the Commission has chosen to take into account. The facts are presented in chronological order, beginning with Jon Sudbø's PhD project, which commenced in 1993. There is an explanation of the raw data underlying parts of

Jon Sudbø's doctorate and several subsequent publications. The Commission discusses in detail which patient data Sudbø actually had or may have had, comparing it with the data Sudbø and his co-authors stated that they have had in different publications. The Commission then reviewed Sudbø's subsequent scientific publications, which are mainly based on the original raw data from the PhD project.

In Chapter 5, the Commission has attempted to illuminate certain circumstances that may help explain how and why things turned out the way they did.

Chapter 6 offers a brief discussion of the possible consequences of the situation, not least for Norwegian research and patients.

Chapter 7 summarizes the findings and the circumstances worthy of criticism which the Commission has found reason to point out. This criticism refers to individuals and institutions alike.

Finally, the Commission has made certain recommendations in Chapter 8 by way of conclusion.

2   The Commission's appointment, terms of reference and method of work

2.1   Appointment of the investigation Commission

On Friday January 13, 2006, the Rikshospitalet-Radiumhospitalet Medical Centre (RR MC) and the University of Oslo informed media that a scientist employed there had admitted to fabrication of data underlying a scientific article in the renowned medical journal The Lancet.

On Wednesday January 18, 2006, the Rikshospitalet-Radiumhospitalet MC and the University of Oslo made it known that they would appoint a special Commission with detailed terms of reference (section 2.3) to perform the investigation and clarify the facts.

2.2   The Commission's composition

The Commission was composed as follows:

- Professor Anders Ekbom, M.D., (Chair), Institusjonen för medisin, Karolinska Universitets Sjukehuset, Stockholm
- Special Advisor Gro E.M. Helgesen, Cand.Pharm., the Research Council of Norway
- Post doc Tore Lunde, LLD, the Faculty of law, the University of Bergen
- Researcher Aage Tverdal, Professor, PhD., the Norwegian Institute of Public Health
- Professor Stein Emil Vollset, PhD, the Department of Public Health and Primary Health Care, the University of Bergen

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- Research fellow Sigmund Simonsen (Secretary), Master of law (Norway), LL.M, the Department of Community Medicine and General Practice, the Norwegian University of Science and Technology.

In addition, the National Cancer Institute (NCI), USA, was offered a seat on the Commission, but has not accepted the invitation.

2.3   The Commission's terms of reference

The purpose of the investigation is to identify and review all factual matters in connection with the research article "Non-steroidal anti-inflammatory drugs and the risk of oral cancer: a nested case-control study" by J. Sudbø et al. in The Lancet, vol 333, pp. 1359-1366, October 15, 2005. The Commission shall also assess other research and other matters considered by the Commission to be related to this case.

The Commission shall make such investigations as it finds necessary to clarify the extent of the breach of standards of scientific research and other criticizable factors.

The Commission shall attempt to clarify whether there are special factors that have influenced what has been done in this case, including the researchers' self interests as well as whether special external interests, frameworks, conditions and tying arrangements for the activity exist.

The Commission shall map and assess any harmful consequences of the research and of other factors included in the terms of reference. This applies to whether this has been harmful in connection with the treatment of patients and if so what harmful effects have ensued. The Commission shall also attempt to map and assess the negative effects caused to the scientific research at the Rikshospitalet-Radiumhospitalet MC and relevant areas of research at the University of Oslo as well as scientific research at other institutions.

The Commission shall assess rules and routines for control and quality assurance which apply to scientific research at the Rikshospitalet-Radiumhospitalet MC and the University of Oslo, and whether these have been complied with in this case. The Commission shall assess whether these rules and routines should be changed, and if so, submit proposals for such changes.

If the Commission during its work should become aware of factors that have effects which should be notified prior to the conclusion of the Commission's work, the Commission must bring this to the attention of the appointing institutions as quickly as possible.

Should the Commission be of the opinion that the terms for reference are limiting the work, this must be brought up with the appointing institutions immediately.

The Commission shall submit its report to the Rikshospitalet-Radiumhospitalet MC and the University of Oslo no later that April 1, 2006, whereupon the report will be made public at the same time as being dealt with independently by the Boards of the two institutions.

2.4 The legal status of the Commission, legal framework, procedural rules, and principles for the investigative process

2.4.1 The Commission's legal status

The investigation commission was appointed by the Rikshospitalet-Radiumhospitalet MC (RR MC) and the University of Oslo (UiO) (hereinafter the "Appointing Institutions") which are, or have been, the employer of the researcher who has admitted to having fabricated research data, and who was the cause of the investigation. This means that the Appointing Institutions are two public institutions.

The Commission is not a "public investigation commission", but rather a temporary and professionally independent administrative body of a special nature, comprised by unbiased experts, created by the management of the aforementioned two public institutions jointly for this case to perform an investigation on their behalf. A fundamental consideration for the Appointing Institutions as well as the Commission has been to have a professionally independent investigation.

There are no particular procedural rules laid down in statute or regulations for this type of ad hoc appointed commissions. In 1968, a committee appointed by the Government submitted a Recommendation for Rules for Investigation Commissions (printed in 1969). The Ministry of Justice then prepared a circular[1] containing a mixture of information, instructions, guidelines, and provisions of a more mandatory nature[2]. The circular is aimed at public investigation commissions appointed by the Government or a ministry, so-called "public investigation commissions". It is therefore obvious that these guidelines are neither prepared with this Commission in mind nor binding for it.

Since the Commission is a body for two public bodies, it is clear, however, that the Commission is subject to the Public Administration Act and general non-statutory requirements as to generally accepted administrative practice. Since the Commission's report is not an individual decision in the terms of the Public Administration Act, only chapters II and III of the Public Administration Act have been of particular importance to the work.

2.4.2   Impartiality and independence

In accordance with the provisions of section 8 of the Public Administration Act, the Commission has considered its own impartiality. No committee member is related to the parties to the case, and the Commission cannot see that circumstances exist that are apt to impair confidence in its impartiality, cf section 6 of the Public Administration Act.

The Commission has placed great emphasis on the consideration of independency in the contact between the Commission and the Appointing Institutions. The Appointing Institutions have made conditions favorable so that is has been possible to carry out the investigation independently, self-contained and without restrictions on the use of resources. Out of consideration for the Commission's independency, its secretariat has not been physically or functionally localized together with the Appointing Institutions, persons or institutions that could have become the subject of investigation. Thus the Commission has made use of premises for the secretariat in Trondheim, and most of the meetings have been held in meeting rooms at Gardermoen *[Oslo Airport]* in addition to telephone meetings. One meeting was held at the Radiumhospitalet in connection with an inspection and talks with employees, and one meeting was held at the Cancer Registry. The Commission has had good framework conditions for carrying out the investigation.

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[1] Rundskriv G-48/75 of March 4, 1975 "Regler for granskningskommisjoner"
[2] Bratholm A. Granskning som statlig virkemiddel for å bringe faktiske forhold eller ansvarsforhold på rene *[Investigation as a governmental tool to bring actual facts or responsibility relationships to light]* . Lov og Rett, 1986: p. 439

2.4.3 Legal measures

The Commission is not authorized to make searches or seizures. People to be interviewed were not under a duty to give any statement to the Commission. The legal basis for obtaining evidence, in the form of oral and written information, including emails and interviews, is consent. All the persons the Commission wanted to interview consented in providing information to the Commission. The same applies to other persons and institutions to which the Commission has addressed inquiries and questions. In general, persons and institutions concerned have collaborated well with the Commission. Nobody refused to collaborate with the Commission.

2.4.4 The duty to clarify the case principle and the sound procedure principle

The Commission has observed the duty to clarify the case principle in section 17 of the Public Administration Act and the principles inherent in requirements as to sound procedure and generally accepted administrative practice. Within the limited time frame available, the Commission has sought to obtain the widest possible information basis, which has included making critical checks of information received from various sources and comparing these against each other with the aim of discovering any discrepancies.

However, the Commission points to the difficulties caused by the fact that some of the circumstances subject to investigation occurred more than ten years ago. Such a comparatively long time span obviously influences the interviewed persons' possibilities to recall details of what took place. The reservations which for these reasons have been necessary to make in relation to what can be deducted from the evidential material, have nevertheless not altered the Commission's main conclusions, as these are stated in the report.

2.4.5 Contradiction

In order to ensure a reliable procedure in line with fundamental considerations of the due process of law and requirements as to generally accepted administrative practice, the Commission has taken as its point of departure, and practiced, the guiding norms laid down in the circular as far as serviceable and natural. The individuals who have been investigated have been notified of this and they have been urged to contribute voluntarily. At the same time, they were informed that they might be subject to criticism and that they in such a case would be notified especially if this would be the case. The individuals who are subject to criticism have also been allowed to read memos, documents and finally the draft report itself, i.e. Chapter 4 through Chapter 7 (Chapters 1 through 3 and 8 contain more general considerations). Thus they have been given the opportunity to respond to the criticism and make contributions at several occasions. The Commission has also met with these persons.

The Commission will here note that the most central person in the investigation, Jon Sudbø, was assisted by his lawyer during the investigation. The procedure and factual matters have been discussed with Sudbø and his lawyer throughout. No substantial objections to the Commission's outlined plan for the investigation as such have been made. In this context the Commission notes that Sudbø's office material has been locked away by his employer during the entire investigation and that Sudbø has been given several offers of access to this material in order to extract documentation and the like of importance to this case. Sudbø has been sent two draft reports together with data lists, connections and other key documents on which the Commission has based itself. Sudbø submitted detailed comments to the first draft – which the Commission incorporated, but did not want to comment on the next draft he received.

In other words, the Commission has arranged for contradiction to a larger extent than it was obligated to do. This was done in consideration of individuals affected, but also in order to illuminate the case as well as possible.

2.4.6   Requirements of proof and thresholds for criticism

The Commission's primary task is to clarify the facts with the aim of discovering whether and to what extent breaches of standards for scientific research and other blameworthy acts have occurred. Like in legal procedures – both civil procedure and criminal procedure – also in investigation procedures there will be different degrees of proof for establishing facts as probable. The Commission has considered what degree of proof should be required in a case of this nature in order for the Commission to rely on particular facts as a basis for criticism.

In the Commission's opinion there are several reasons why a more stringent degree of proof should apply than the traditional principle in civil procedure of proof by a so-called simple preponderance of probability[3]. In the first place reference is made to the fact that the object of the investigation is to clarify facts with the intention of discovering the extent of scientific dishonesty and the like. Criticism on such a basis must be considered highly invasive for the individuals involved. Taking into account the serious legal consequences and any sanctions that may be triggered by conclusions that scientific dishonesty, breach of generally accepted research practice and similar criteria exists, the ordinary principle of proof by a preponderance of probability must be deviated from in favor of the person concerned by the investigation. In the second place, in an investigation process the production of evidence and practice of the principle of immediacy will be more limited than during an ordinary court procedure. On the other hand, the Commission finds that there is no basis for requiring a degree of proof that is as strict as in criminal law. Based on an overall assessment, the Commission finds that the degree of proof to be applied in order for it to rely on a particular fact as proven should be proof by a so-called qualified preponderance of probability. The Commission has applied this principle in its investigation and preparation of the report.

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[3] See in more detail Skoghøy JE. Tvistemål *[Civil Procedure]* Oslo: Universitetsforlaget, 2001: p. 673 et seq.

The Commission has therefore, mainly out of regard for the due process of law, but also for pragmatic reasons, applied a very high threshold for criticism of persons. Many individuals have been involved in the research which has been subject to investigation, and, in the Commission's view, any criticism for less serious acts may have a disproportionate effect on individuals, especially seen in the light of the Appointing Institutions' express intention to publish the report as well as the extensive press coverage this case has been the subject of.

In relation to the institutions involved, however, the Commission has found grounds for a somewhat different approach. The Commission has notified two institutions that they might be subject to criticism. These institutions have been given the opportunity to read the criticism itself, but not the draft report in its entirety, and have thus been given a limited possibility to contribute. Furthermore, the Commission has chosen not to notify the Appointing Institutions: the Rikshospitalet-Radiumhospitalet MC and the University of Oslo, although these as responsible institutions must suffer criticism. Notification has been omitted to prevent the risk of any unfortunate influence from these institutions. However, the Commission has had meetings with managers at several levels at the Rikshospitalet-Radiumhospitalet MC and the University of Oslo in order to clarify factual matters. The omitted notification and possibility to read the draft report are also related to the institutions' relative strength compared to individuals. The consideration for the Commission's integrity and professional independency has also been an important element in this assessment. The Commission has considered it such that the institutions to a completely different degree than individuals must put up with public criticism.

2.4.7 The presumption of innocence

The so-called presumption of innocence embodied in the European Convention on Human Rights (EHRC) Article 6 (2) states that "everyone charged with a criminal offence shall be presumed innocent until proved guilty according to law". The Convention has been incorporated into Norwegian law, cf the Human Rights Act. The Commission's terms of reference have been established with the aim of clarifying factual matters particularly related to the Lancet article, and discovering the extent of breach of standards for scientific practice. The formulation of the terms of reference implies that the Commission may perform its tasks without making findings of guilt that violate the presumption of innocence.

2.4.8 Publicity

The Commission is an administrative body in the terms of section 1 of the Freedom of Information Act. The Freedom of Information Act thus applies to the Commission's work. All documents

supplied to the Commission are subject to disclosure unless the grounds for exemption in sections 4, 5, 5a or 6 apply. Recording of statements, draft reports and similar notes are considered internal documents which may be exempted pursuant to section 5. This also applies to contributions and support documents supplied to the Commission by persons who have given statements, as well as in-depth comments they have given to print-outs sent them for perusal. Voluntary public access has not been given to statements recorded during the investigation. The Commission has received one request for access to documents, by Verdens Gang [*a major Norwegian daily newspaper*]. The request was refused pursuant to section 4 (1) of the Freedom of Information Act. Following a complaint from Verdens Gang and a renewed assessment, certain documents were released.

When preparing the report, the Commission has had in mind the Appointing Institutions' express aim to make the report available to the public. Annexes that document errors and defects in the patient material and that contain patient-identifiable information (in the form of block numbers, for example) have been exempted from disclosure and have only been submitted to the Appointing Institutions and the persons and institutions against which the criticism has been directed and which in that capacity previously have been dealing with these data. An anonymized and simplified version of these annexes, without patient-identifiable information, has been included as an annex to the report which will be published.

The Commission will be dissolved after the submission of the report to the Rikshospitalet-Radiumhospitalet MC and the University of Oslo, and the documents in the case will then be handed over to and managed by the Rikshospitalet-Radiumhospitalet MC and the University of Oslo for filing in the normal manner. The material will be subject to the Freedom of Information Act and the legislation on archives. Any subsequent right of access may, moreover, be limited due to restrictions which may follow from consents given, secrecy rules and the like.

2.5 The Commission's relation to the terms of reference

The terms of reference are stipulated very broadly. The formulation of the terms of reference must be seen in the light of the fact that the Appointing Institutions at the time of appointment obviously did not have a full view of the more detailed nature of the case and its extent, and that one did not want to place restrictions on the Commission's investigation.

Furthermore, the Commission has noted the extensive press coverage and debate which followed in the wake of this case, including more or less explicit expectations to the Commission held by commentators and others. Obviously, the Commission has neither tried nor had any possibility to accommodate all such expectations.

However, in line with its terms of reference, the Commission started out broadly and considered a series of relevant factors. At the same time it was evident that it would have been an insurmountable task to make an equally thorough assessment of all the questions raised in the terms of reference within the limited time frame at the Commission's disposal. The Commission has therefore had to make continuous

delimitations and definitions of the terms of reference. The Commission has then had to prioritize those factors that in the Commission's view have appeared as the most central and serious.

In the terms of reference the Commission was asked to submit its report to the Appointing Institutions no later than April 1, 2006. The Commission gradually saw that it would not be possible to comply with this deadline. A new deadline was fixed at June 30, 2006 according to the Commission's own suggestion.

2.6   In more detail about the Commission's method of working

2.6.1   In general

The members of the Commission have performed their assignment in addition to other tasks. The Commission's secretary has worked full time for the Commission and also had a 20% position at the NTNU. Furthermore, the Commission has received assistance with office work, i.a. for transcription of interviews, from Marit Kvidal and Toril Synnøve Strand. All have signed a statement on confidentiality.

2.6.2   Meeting activity

The Commission has held 13 all day meetings and 11 telephone meetings. A fair amount of the meeting time has been spent to meet key persons – altogether 15.

2.6.3   Retrieval of information

The Commission's principal task has been to clarify the facts. The Commission has obtained written as well as oral information. The scope of information is considerable. The written material is stated in document lists included as annexes to the report. The material can be grouped into 1) research publications in which Jon Sudbø was the first author or coauthor, 2) correspondence, including email between the Commission and persons, institutions and public authorities involved, and 3) data files containing information on background material used, including data files prepared by the Commission with the aim of reconstructing and checking the background material. All information has partly been recorded on tape and then transcribed, partly received per telephone and recorded in internal case documents and/or exchanged per email to the members of the Commission, and partly received in meetings, without tape recordings having been made, but where information has been recorded in internal case documents. The Commission has used information relevant to the terms of reference only.

It is inherent in the terms of reference that it is research under the auspices of Jon Sudbø that has been at the centre of the work, because Jon Sudbø, based on the information existing as per the time of the

appointment, already had admitted to fabricating research data as regards an article in The Lancet from October 2005.

Consequently, the Commission decided early on, in view of the nature of the case, that the entire scientific activity and production by Jon Sudbø, which according to the data base PubMed[4] comprise 38 publications, had to be investigated, see the publication list, Annex 1. A more detailed account of this is given in the review of the facts in Chapter 4.

This meant that the authors who had contributed to publications together with Jon Sudbø (approximately 60) in fact also have been subject to investigation. These persons have been treated equally. The authors were notified in writing that formally they were subject to investigation, and formally notified that this could result in criticism. At the same time, they were asked to make a written statement voluntarily. They were also informed that they were under no duty to give such a statement, since the Commission had no authority to *order* statements from anyone. The authors all responded to the Commission's request. In addition, certain persons and collaborators who are thanked in *Acknowledgements* were contacted. The Commission has also asked additional and follow-up questions as needed. Several authors and collaborators have also given oral statements before the Commission (15 persons). Jon Sudbø has made oral and written statements to the Commission. With the understanding of the persons interviewed, most of the statements have been recorded on tape and transcribed. Those who have given statements have been given the opportunity to read the transcripts from the conversations, and submit corrections, definitions or additional comments. In accordance with the conditions for tape recording stipulated by the interviewed persons themselves, the tapes have been deleted by the Commission.

The Commission has also obtained written and oral information from relevant bodies and institutions with which Jon Sudbø has been in contact in connection with his research. Particular mention should be made of the Cancer Registry of Norway, which itself has performed very extensive investigations as part of its own internal control, i.a. because the Cancer Registry allegedly has been a key cooperation partner for Jon Sudbø. The Cancer Registry's independent investigations have been of great value to the Commission, first and foremost because the Cancer Registry has access to most of the background data used by Sudbø in his research.

As accounted for in detail in Chapter 4, the key information carrier has been available data lists and published research results which could illuminate the background material which forms the basis for Jon Sudbø's research. These data files have then been correlated and compared with each other and other available documentation, i.a. from the Cancer Registry. In this way, the Commission has tried to

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[4] www.pubmed.gov

recreate the actual background material which has or with a high degree of probability has been used by Jon Sudbø in his research.

2.6.4   Good scientific practice, norm deviation and dishonesty

As already stated, one object of the investigation has been, through a clarification of the facts, to discover whether any breach of good scientific practice, i.e. breach of good research practice, has occurred. The Commission has subsequently delimited this object to apply to *gross* and *serious* breaches. This means first and foremost "scientific dishonesty", as this has been defined traditionally, as well as more serious degrees of negligence and blameworthiness. The criterion "scientific dishonesty" has recently been enacted in the Act on Research Ethics (not yet in force) in which section 5 (2) reads:

> "Scientific dishonesty means falsification, fabrication, plagiarism and other serious breaches of good scientific practice perpetrated with intent or gross negligence in the planning, implementation or reporting of research."

In view of the fact that the requirement of guilt for scientific dishonesty is formulated as a requirement of intent or gross negligence, the Commission has applied a relatively high threshold for finding breaches of scientific honesty, as well as gross and serious breaches of good research practice. The Commission also refers to section 3.2. On this basis, the Commission has not considered it appropriate to make a detailed investigation of each one of the 60 authors, with the aim to discover deviation from norms which must be considered to be of less importance in relation to the main issue in this case.

This important delimitation is first and foremost justified by the fact that such less serious cases are in an entirely different category from the serious allegations which it has been the Commission's primary task to investigate. It should be underlined that this must not in any way be construed as an attempt by the Commission to play down petty offences. On the contrary, the prevention of any form of norm deviation from good research practice has been given great emphasis in the Commission's recommendations. The delimitation must rather be seen in light of a real and everyday need to establish a practicable framework for the Commission's work. Within the Commission's restricted time frame it has not been possible to investigate each individual author in detail, with the requirements as to thoroughness and due process this would have entailed. The Commission here refers to the fact that each individual person who the Commission might have found reason to criticize directly, regardless of whether trivial or serious acts were involved, would have had to be given the opportunity to give a statement and refute any criticism directed at him/her (the contradiction principle). Finally, the Commission emphasises that the work connected to

discovering the most grave and serious acts has been much more extensive than what one had reason to predict in the beginning.

On the other hand, the Commission has found reason to present certain more general remarks related to how researchers and research institutions should act, including the lesser norm deviations which seem to have occurred. Thus, the criticism of persons is first and foremost related to gross and serious norm deviations, whereas the detection of less serious matters has been restricted to a more general and institutional level.

Accordingly, two questions have been at the centre of the Commission's clarification of the facts:

1. Have gross and serious breaches of good research practice taken place, and if so, to what extent?
2. What happened (causal factors), and who is responsible for any breaches of good research practice?

Before replying to these questions, the Commission has found reason to outline the general framework and the background against which this specific case has been assessed, see Chapter 3.

3. Regulation of medical research

3.1 Overview of the applicable set of rules

The second section of the terms of reference reads:

> "The Commission is to make such investigations as it finds necessary to clarify the extent of
> breaches of standards for scientific research and other blameworthy matters."

"Breaches of standards for scientific research and other blameworthy matters" is a very broad concept. The concept aims at acts *and* omissions which are in breach of established norms of operation/rules of operation – here: good research practice. Such norms or rules for accepted conduct may have different status and designation, as for example:

- Ethical norms and social norms[5]
- Vocational norms (researchers and medical staff's self regulations)[6]
- Instructions and norms laid down by the employer
- Legal norms (statutory and non-statutory legal rules)[7]

The regulation of medical and health related research is marked by an intimate interaction between ethics, law and the profession's own norms, where the ethics (ethical reflections) normally forms the basis for legal norms as well as the profession's own norms.

The Commission will not here give a detailed description of all relevant rules that apply to medical research. These rules have recently been described comprehensively in other presentations, and the Commission would refer to these.[8]

However, the Commission will very briefly outline the existing legal framework against which this specific case has been assessed. The framework defines the expectations and requirements made to Norwegian researchers and research environments.

Although, basically, research is to be free and independent, there are of course rules of the game which researchers must follow in the same manner as everybody else.

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[5] See for example Ruyter KW. Forskningsetikk *[Research Ethics]*. Oslo: Gyldendal, 2003
[6] See for example Benestad HB, Laake P. Forskningsmetode i medisin og biofag *[Research method in medicine and bio subjects]* Oslo: Gyldendal, 2004
[7] See for example Simonsen S, Nylenna M. Helseforskningsrett *[Health Research Law]* Oslo: Gyldendal, 2005
[8] NOU *[Norwegian Official Reports]* 2005:1: God forskning – better health *[Good Research – Better Health]*. Act relating to medical and health research that involves humans, human biological material and health data (the Health Research Act); Ot.prp. *[Proposition to the Odelsting]* no. 58 (2005-2006) On an Act relating to the dealing with ethics and honesty in research; Ruyter KW. Forskningsetikk *[Research Ethics]*. Oslo: Gyldendal, 2003; Benestad HB, Laake P. Forskningsmetode i medisin og biofag *[Research method in medicine and bio subjects]* Oslo: Gyldendal, 2004; Simonsen S, Nylenna M. Helseforskningsrett *[Health Research Law]* Oslo: Gyldendal, 2005

There are a series of rules and control routines for medical and health related research. Traditionally, the research community has regulated itself, via the development of norms for good scientific practice (professional norms and sector practice). In later years however, the legislative authorities have also played a more active role, particularly as regards medical and health related research that involves people, human biological material and/or personal data.

The Patients' Rights Act, the Biobanks Act and the Personal Data Act are key examples of statutory legal rules. Non-statutory legal rules come in addition. A series of written and unwritten professional norms (self regulations), as for example the Helsinki declaration prepared by the World Medical Association (WMA) and the so-called Vancouver rules, cf section 3.5, are moreover still highly relevant. More detailed working instructions or implied requirements as to acceptable conduct at the individual research institutions come in addition. These are currently available at the institutions' intranet. An increasing amount of international directives and conventions are also influencing the regulation of medical and health related research, and contributes, i.a., to many similarities between the national regulations in various countries. The EU Personal Data Directive 1995 and the Medicine Directive 2001, as well as the European Council's Convention on Biomedicine and Human Rights 1997, are examples of the latter.

A public committee which reported on the regulation of medical research, *the Nylenna Committee*, found that a lack of regulations was not the primary problem, although certain flaws existed.[9] In the committee's view, the main problem was that the regulations were fragmented, complex and inaccessible, and that very few people for that reason seemed to have satisfactory overview of the set of rules.

In order to obtain an overview of the current set of rules for research, it may be appropriate to differentiate between various types of norms of operation for medical and health related research, based on the purpose of the norms (although these obviously must be seen in connection):

- **Protective rules:** These concern rules aiming at protecting the integrity of the individual person (research participant).

   The main rules are that consent by the individual research participant (i.e. the person participating in the research directly or indirectly, i.a. by giving tissue samples or personal data) must exist. In addition, the research must be sound and in line with good research practice, as well as be assessed in advance by one of the regional committees for Medical Research Ethics (REK) and other relevant bodies.

   The regulations do not only comprise research on humans, but also the use of exclusively human biological material and personal data. Violation of these rules will often be considered as serious,

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[9] NOU *[Norwegian Official Reports]* 2005:1: <u>God forskning – better health</u> *[Good Research – Better Health]*. Act relating to medical and health research that involves humans, human biological material and health data (the Health Research Act)

because they jeopardize other people's life and health, or infringe their integrity (private life). These regulations are therefore often a result of statutory provisions or non-statutory law.[10]

The Human Rights Act 1999, the Medical Personnel Act 1999, the Patients' Rights Act 1999, the Specialist Health Services Act 1999, the Biobanks Act 2001, the Personal Data Act 2000 and the Personal Health Data Filing System Act 2001 are key statutes in this field. The so-called Helsinki Declaration 1964[11] indicates principal professional norms. The legislation relating to the protection of animals comes in addition in the case of experiments on animals.

- **Rules for scientific integrity**: This concerns rules aiming at regulating the research itself, as for example norms for the choice of method, design of studies and the like, which are to ensure that research results are valid and that the knowledge can be generalized. It is therefore fundamental that data should not be manipulated, fabricated or falsified. Furthermore, the inclusion of research participants must not be unduly selective, and research data must be stored for some time after completion in order to secure the opportunity to check. Honesty, thoroughness, completeness and openness are key ideals here.[12]

  Violation of one or several of such norms of conduct/rules (good scientific practice) may entail that the research results cannot be considered as valid.

  These rules are primarily unwritten and follow from established scientific practice and good research practice as well as more general requirements to soundness.

- **Publication rules (integrity rules):** A third group of regulations are those applying to the publication of research results. This concerns rules that are to contribute to openness around and opportunity to check research results, i.e. that what is written in the publication is in fact correct and adequate, such that others can use it as a basis for their further work. That which speaks for and against (positive as well as negative) results must be stated. Conflicts of interest, associations and the like which can be imagined to have influenced the results, should also be stated. Another dimension,

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[10] NOU *[Norwegian Official Reports]* 2005:1: God forskning – better health *[Good Research – Better Health]*. Act relating to medical and health research that involves humans, human biological material and health data (the Health Research Act); Simonsen S, Nylenna M. Helseforskningsrett *[Health Research Law]* Oslo: Gyldendal, 2005; Ruyter KW. Forskningsetikk *[Research Ethics]*. Oslo: Gyldendal, 2003
[11] The Helsinki Declaration: World Medical Association Declaration of Helsinki. Ethical Principles for Medical Research Involving Human Subjects. Ferney-Voltaire: World Medical Association, 2004.

[12] Benestad HB, Laake P. Forskningsmetode i medisin og biofag *[Research method in medicine and bio subjects]* Oslo: Gyldendal, 2004. Tranøy KE. Vitenskapen – samfunnsmakt og livsform *[Science – community power and way of life]*. Oslo: Universitetsforlaget, 1986.

which can also be said to belong in this "group of rules", is rules and practice concerning authorship, plagiarism and copyright.[13]

These rules also primarily follow from established scientific practice and good research practice, as well as more general requirements as to soundness. The Copyright Act 1961 and the so-called Vancouver rules may also be relevant in this context.

3.2   Different degrees of norm deviation, guilt and blameworthiness

It is important to emphasize that deviations from norms and breach of rules occur in many shades – from the trivial to the conspicuous.[14]

In the recently enacted Research Ethics Act (not yet in force) section 5 (2), the so-called *scientific dishonesty* is restricted to certain gross and serious deviations, i.e. "falsification, fabrication, plagiarism and other serious breaches of good scientific practice perpetrated with intent or gross negligence".

Obviously also "less serious" deviations occur, which may nevertheless represent a breach of good research practice, as for example flawed source references, failing design, breach of quality assurance routines, misleading authorship, or by "disregarding" extreme or unexpected observations and other oddnesses which do not entirely agree with one's own hypotheses. Such deviations must also be taken seriously, since they are suited to impair the quality and trustworthiness of the research, and to create a climate for more serious deviations.

Thus there is a sliding transition from the trivial to the gross and more serious deviations.

In the same way, the degree of guilt will vary, from excusable mistakes via cases in which one has acted unintentionally but nevertheless should have acted differently (negligence), to willful breach of the rules, knowingly committed. Norm deviations may thus be criticizable and blameworthy even if the researcher has not acted knowingly, but maybe rather been negligent, uninterested, careless, incompetent or the like.

The degree of blameworthiness will thus depend on the degree of the norm deviation and guilt.

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[13] Ot.prp. *[Proposition to the Odelsting]* no. 58 (2005-2006) On an act relating to the dealing with ethics and honesty in research. NOU *[Norwegian Official Reports]* 2005:1: God forskning – better health *[Good Research – Better Health]*. Act relating to medical and health research that involves humans, human biological material and health data (the Health Research Act). Simonsen S, Nylenna M. Helseforskningsrett *[Health Research Law]* Oslo: Gyldendal, 2005. Ruyter KW. Forskningsetikk *[Research Ethics]*. Oslo: Gyldendal, 2003. Benestad HB, Laake P. Forskningsmetode i medisin og biofag *[Research method in medicine and bio subjects]* Oslo: Gyldendal, 2004.

[14] Nylenna M, Simonsen S. Scientific misconduct: a new approach to prevention. The Lancet 2006;367:1882-1884.

3.3 Personal liability and overall system responsibility

The main rule in Norwegian law is that an individual is liable for his/her acts and omissions. Thus the individual person may be held personally liable for what he/she has done, alternatively not done, but ought to have done, and be met by different sanctions. This follows from general principles of non-statutory negligence liability and more special liability rules, among other things.

The large majority of researchers, however, are ordinary employees in public or private sector. This means that in addition, it may be question of a system liability for the employee's superior, i.e. the person/organisation who is liable for the person who actually performs the act. Personal liability does not exclude system liability, and vice versa.

Basically, employers are liable for the acts of their researchers, and the main rule is that they are liable for the acts of their employees, irrespective of whether the employer is to blame or not. This so-called employer liability follows from section 2-1 of the Act relating to Compensation for Claims, of which subsection 1, first sentence, reads:

> An employer is liable for damage caused willfully or negligently during the employee's performance of work or assignments for the employer, taking into account whether the expectations the parties sustaining the loss reasonably can make to the activity or service have been neglected.

For example, research is stated as one of the main tasks of the specialist health service on the same level as medical treatment, cf section 3-8 no 3 of the Specialist Health Services Act. The requirements in the Specialist Health Services Act relating to soundness, organization and management at different levels will thus apply to research. The medical centers have a hierarchic system, with the state as owner, having in principle the superior responsibility as well as the managerial prerogative and right to instruct, cf sections 3 and 7 of the Health Enterprises Act.[15] However, the state has appointed a board of directors, which again has appointed a general manager, a CEO, having the day-to-day responsibility and managerial prerogative and right to instruct, cf section 37 of the Health Enterprises Act. But this responsibility, including the managerial prerogative and right to instruct, will be delegated downwards and distributed to clinical managers, department managers, sections heads and project managers (including project managers for research projects, i.e. the person having the day-to-day responsibility for a specific research project).[16] Section 3-9 of the Specialist Health Services Act states that there shall be a responsible manager at each level. But such hierarchic delegation (line management) does not mean that the superior person in the line ceases to be liable. In principle, nothing prevents the overall liability to be divided, for example between a medical centre and a

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[15] Buskop T. <u>Hvem har ansvaret for et forskningsprosjekt?</u> [Who is responsible for a research project?] www.forskningsjus.no. 2006.
[16] Simonsen S, Nylenna M. <u>Helseforskningsrett</u> *[Health Research Law]* Oslo: Gyldendal, 2005

university. In such cases, however, it will normally be a question of joint and several liability, i.e. that both institutions are liable irrespective of each other – one for all, all for one. In general it must be assumed that the institution to which the day-to-day research has its closest relation, typically the hospital, carries the primary responsibility for the research.

Preparation and sound organization of the research, stating responsibilities and such like, are thus key tasks for the employer, i.e. the research institution. Where research is concerned, proper account must of course be taken of the customary academic freedom, i.e. that the employer must not in any undue manner try to influence the research. The employer may nevertheless not provide employed researchers with unlimited authority and disclaim any liability. Thus the employer may also be held liable on an independent basis, for example due to lack of routines, training, management, control and supervision in connection with research as well as medical treatment. This applies in particular when patients, patient material, patient data, animals or other sensitive research objects are involved in the research.

The current Act relating to Universities and Colleges of April 1, 2005, states explicitly in section 1-5 that universities and colleges may not be instructed regarding the academic contents of their teaching and the content of research or artistic or scientific development work. In evaluations of Norwegian research a stronger professional management is at the same time called for. Professional management and management structures may establish frameworks for research to be performed by the individual employed scientist. Tension may therefore exist between the individual person's academic freedom and the institution's professional management responsibility at all levels, even if the act does not contain provisions that directly can be said to restrict the individual academic freedom in an unfortunate way.

The recommendation, Innst.O. nr 70 (2005-2006), from the parliamentary standing committee on church, education and research concerning the Act on Research Ethics, states:

> The committee takes as its point of departure, as did the Government, that research takes place under a considerable degree of freedom and trust, and thereby also a considerable degree of personal responsibility for the individual researcher. At the same time, there is reason to underline that the research institutions have an independent responsibility for control and management. However, the institution's professional management responsibility must continuously be assessed against the concern for academic freedom and the individual scientific employee's rights. The committee has noted that this issue will be discussed by the so-called Underdal committee, which is to submit its recommendation in October 2006.

It should be noted here that employed researchers, in spite of these formal points of departure, traditionally have had an extremely free role at most of the public research institutions, among other things indeed to secure the professional independency of research. However, it should be noted that there is not necessarily a contrariety between professional integrity and independency, and an overall responsibility for and supervision of the institution's activity being sound.

A need to raise awareness of the research institutions' responsibilities and duties is a common subject in reports on the regulation of medical and health research.[17]

Supervisors in PhD or master degree projects may have different roles, and do not necessarily form part of research institutions' line management. In clinical research, the supervisor will often also be a co-researcher, and then usually a project manager, so that this person holds the day-to-day responsibility for the specific research project. The supervisor will then have an overall line responsibility for the PhD candidate or the student. But the supervisor may also have a more retired role by functioning solely as an advisor and conversation partner (mentor). In such cases, the supervisor's responsibility will be more modest and derivative. The role as supervisor is discussed in more detail in section 3.7.

3.4   *The application of the rules in time*

A basic principle of the due process of law is that the rules prevailing at the time when the act of omission occurred shall apply.

Because circumstances in this specific case span a period from 1993 to 2006, it has been important to the Commission to clarify the rules in force at all times.

As a point of departure, one may say that the current main principles, as presented above, have been unchanged since 1993 when John Sudbø started his PhD project and his scientific career. Scientific dishonesty was, in other words, as unacceptable then as now.

In this connection, the Commission has obtained statements from the Regional Committee for Medical Research Ethics-South, the Data Inspectorate, the Norwegian Social Science Data Services and the Directorate for Health and Social Affairs. These bodies clearly state that the current rules in the areas that have been relevant in this case in all essentials correspond to the rules and principles existing and being practiced in 1993. As an example, the requirements as to a licence for, and an advance assessment of, research projects by the Data Inspectorate and the Regional Committee for Medical Research Ethics respectively applied then as now. The same was true for the requirement for either a participant consent or dispensation from the duties of secrecy by the Directorate for Health and Social Affairs for the use of patient information in research subject to a duty of secrecy. Earlier, prior to January 1, 2002, this public authority task was vested in the Norwegian Board of Health.

On the other hand, a certain tightening of the rules at the more detailed level, as for example the rules on the protection of personal data, has taken place. The most important is perhaps an increasingly raised awareness surrounding the rules that apply among researchers, institutions and public bodies.

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[17] NOU *[Norwegian Official Reports]* 2005:1: <u>God forskning – better health</u> *[Good Research – Better Health].* Act relating to medical and health research that involves humans, human biological material and health data (the Health Research Act)

The employees at the Rikshospitalet-Radiumhospitalet MC and the University of Oslo have also been given increasingly more and more defined internal rules and instructions to relate to at the work place than they had previously.

3.5   *In particular about authorship*

3.5.1   Some points of departure

The Commission sees the need for an introductory presentation of rules and practices related to issues linked to authorship on a more general level before commenting on individual circumstances.

Under this section, the Commission will first refer to the fact that the discussion on authorship, coauthorship and contributors within medical research is an old and continuously recurrent subject matter that has been the object of extensive discussions internationally for years. The question of who is the legitimate author of an article is one of the most discussed and controversial questions within medical publishing.[18]

Several arguments may be pleaded in favor of uniform rules for authorship.

In the first place the responsibility to readers including the scientific community presupposes that the person or persons stated as authors in fact can defend the message presented.

In the second place various requirements to and different practicing of rules relating to authorship may give an incorrect and unjust basis for comparisons within the system of merit in which scientific authorship plays the lead. One aspect in this connection is also funding systems within scientific research,

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[18] In respect of this discussion, refer to, *i.a.,* Bates T, Anič A, Marušič M, Marušič A. Authorship Criteria and Disclosure of Contributors. Comparison of 3 General Medical Journals With Different Author Contribution Forms. JAMA 2004; 292: 86–88; Bhopal R et al. The vexed question of authorship: views of researchers in a British medical faculty.BMJ 1997; 314: 1009; Hoen WP, Walvoort HC, Overbeke AJPM. What Are the Factors Determining Authorship and the Order of the Authors' Names?, A Study Among Authors of the Nederlands Tijdschrift voor Geneeskunde (Dutch Journal of Medicine). JAMA 1998; 280: 217–218; Kwok LS. The White Bull effect: abusive coauthorship and publication parasitism. J Med Ethics 2005; 31: 554–556; Nylenna M. Forfatterskapskriteriene er endret *[The authorship criteria are changed].* Tidsskrift for Den Norske Lægeforening 2000; 120: 1844; Pignatelli B, Maisonneuve H, Chapuis F. Authorship ignorance: views of researchers in French clinical settings. J Med Ethics 2005; 31: 578–581; Rennie D, Flanagin A, Yank V. The Contributions of Authors. JAMA 2000; 284: 89–91; Sheikh A. Publication ethics and the research assessment exercise: reflections on the troubled question of authorship. Journal of Medical Ethics 2000; 26: 422–426; Sox HC. Research Misconduct, Retraction, and Cleansing the Medical Literature: Lessons from the Poehlman Case. Annals of Internal Medicine 2006; 144: E-7–E-11; Yank V, Rennie D. Disclosure of Researcher Contributions: A Study of Original Research Articles in The Lancet. Annals of Internal Medicine 1999; 130: 661–670.

where authorship is one criterion in relation to the granting of research support. In later years, the financial incentives related to such authorship have been reinforced by governments.

3.5.2 The Vancouver rules for medical publication

The so-called Vancouver rules or criteria[19] are at the centre of the discussion on authorship within medical and health research. These standardized criteria were prepared under the auspices of a small group of editors of international medical journals, who met informally in Vancouver in 1978, with the aim of establishing guidelines for manuscripts delivered to the publications. The group, gradually becoming known as the Vancouver Group, published the guidelines initially in 1979. The Vancouver Group expanded and gradually developed into the International Committee of Medical Journal Editors (ICMJE) meeting annually. The criteria have been subject to continuous revisions. The Vancouver Group has been a small group without any real formal or legal status. In spite of this, the Vancouver Group has worked up an authoritative status enjoying the respect of researchers, academic institutions and public authorities globally. The authoritative force of the Vancouver rules can to a large degree be ascribed to the important medical journals represented in the Group, among them the American New England Journal of Medicine and Journal of the American Medical Association (JAMA), as well as the British Medical Journal (BMJ) and The Lancet.

The fundamental idea behind the Vancouver Group's criteria for authorship is that authorship is an intellectual activity and that the ideas, analyses and not least the preparation of manuscript itself are the core of the scientific authorship.[20]

This idea is in good harmony with the more general criteria for authorship and general copyright principles. It should be noted, however, that the Vancouver rules are not more stringent than more general criteria. On the contrary, the Vancouver rules have been subject to continuous adaptations which make them currently appear as relatively liberal, in the sense that for example data retrieval is now equal to idea/design and analysis/interpretation. By the revisions of the last 10 years, the Vancouver rules have also downgraded the responsibility to be borne by the individual author, see Annex 2. Whereas in 1997 they stated that "Each author should have participated sufficiently in the work to take public responsibility for the content", later versions state that "Each author should have participated sufficiently in the work to take public responsibility for appropriate portions of the content". The 2003 version stated that "one or more authors shall take responsibility for the integrity of the work as a whole, from inception to published article". In the 2006

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[19] Uniform Requirements for Manuscripts Submitted to Biomedical Journals: Writing and Editing for Biomedical Publication – updated February 2006

[20] Nylenna M. Forfatterskapskriteriene er endret *[The authorship criteria are changed]*. Tidsskrift for Den Norske Lægeforening 2000;120:1844

version this responsibility is further weakened to "Some journals now also request that one of more authors, referred to as "guarantors", be identified as the persons who take responsibility for the integrity of the work as a whole, from inception to published article, and publish that information".

Thus the Vancouver rules may be considered as a definition of the more general authorship principles which apply generally in all fields. In other words, the criteria are adapted to the particular circumstances one believes exist in relation to medical research and publication of medical research results in medical journals. The authorship criteria are thereby not necessarily characteristic of the principles applying outside the medical professional area. In this context, the Commission in particular notes the new amendments of 2000 following a critical revision, in which to collect data was assigned a value, which together with writing and approving the manuscript may lead to authorship. The fact that supplying data material may qualify for authorship is, in the Commission's view, related to this being widespread in the medical professional community, and that it is often a precondition for being able to perform medical and health research. To a large degree one is dependent on sub-suppliers who do not necessarily participate in the intellectual process of the research project and/or the publication of the research results but who nevertheless have a substantial role in the research project. They are necessary contributors for the research project being able to be implemented. This contribution and the expertise held by the sub-suppliers must in one way or another be rendered visible and valued. These factors, in combination with medical publications also having become an important element in the medical community, have resulted in a practice in which persons are credited for their efforts in the research projects through a coauthorship, without having necessarily contributed to any particular degree to the intellectual process and creation of the intellectual work itself which one associates with authorship in general. In many important medical studies it is not unusual that an article has from 20-50 coauthors. The typical contribution, which is in full compliance with the Vancouver rules, can then be contributions with patient or other data material as well as a critical review of a final manuscript and its approval. For many people, and then in particular the general public, it may appear as rather incomprehensible that one departs from the general perception of the authorship concept.

The authorship criteria stated in the Vancouver rules must be seen in light of medical research often being characterized by collaboration projects. One is often collaborating across professional areas, for example laboratories and statisticians collaborating with clinicians and epidemiologists. Cooperation also takes place across institutions, and not least across national borders. This leads to a split of areas of work and responsibility. These factors have been at the centre of the debate linked to coauthorship.

But such distribution of tasks may also contribute to pulverizing the responsibility. The Vancouver rules have taken this into consideration in that publication under the auspices of large research groups shall include one or several authors/coauthors assuming the primary responsibility for the publication and the project as a whole, in the same way as the project manager will have the primary responsibility for the planning, implementation and completion of the research project as a whole.

For the sake of completeness, the Commission finds is appropriate to include the key provisions in the Vancouver rules in their entirety, as they read per June 1, 2006:

"II. Ethical Considerations in the Conduct and Reporting of Research

II.A Authorship and Contributorship

II.A.1. Byline Authors

An "author" is generally considered to be someone who has made substantive intellectual contributions to a published study, and biomedical authorship continues to have important academic, social, and financial implications. (1) In the past, readers were rarely provided with information about contributions to studies from those listed as authors and in acknowledgments. (2) Some journals now request and publish information about the contributions of each person named as having participated in a submitted study, at least for original research. Editors are strongly encouraged to develop and implement a contributorship policy, as well as a policy on identifying who is responsible for the integrity of the work as a whole.

While contributorship and guarantorship policies obviously remove much of the ambiguity surrounding contributions, it leaves unresolved the question of the quantity and quality of contribution that qualify for authorship. The International Committee of Medical Journal Editors has recommended the following criteria for authorship; these criteria are still appropriate for those journals that distinguish authors from other contributors.

• Authorship credit should be based on 1) substantial contributions to conception and design, or acquisition of data, or analysis and interpretation of data; 2) drafting the article or revising it critically for important intellectual content; and 3) final approval of the version to be published. Authors should meet conditions 1, 2, and 3.

• When a large, multi-center group has conducted the work, the group should identify the individuals who accept direct responsibility for the manuscript (3). These individuals should fully meet the criteria for authorship defined above and editors will ask these individuals to complete journal-specific author and conflict of interest disclosure forms. When submitting a group author manuscript, the corresponding author

should clearly indicate the preferred citation and should clearly identify all individual authors as well as the group name. Journals will generally list other members of the group in the acknowledgements. The National Library of Medicine indexes the group name and the names of individuals the group has identified as being directly responsible for the manuscript.

• Acquisition of funding, collection of data, or general supervision of the research group, alone, does not justify authorship.

• All persons designated as authors should qualify for authorship, and all those who qualify should be listed.

• Each author should have participated sufficiently in the work to take public responsibility for appropriate portions of the content.

Some journals now also request that one or more authors, referred to as "guarantors," be identified as the persons who take responsibility for the integrity of the work as a whole, from inception to published article, and publish that information.

Increasingly, authorship of multi-center trials is attributed to a group. All members of the group who are named as authors should fully meet the above criteria for authorship.

The order of authorship on the byline should be a joint decision of the coauthors. Authors should be prepared to explain the order in which authors are listed.

II.A.2. Contributors Listed in Acknowledgments

All contributors who do not meet the criteria for authorship should be listed in an acknowledgments section. Examples of those who might be acknowledged include a person who provided purely technical help, writing assistance, or a department chair who provided only general support. Editors should ask authors to disclose whether they had writing assistance and to identify the entity that paid for this assistance. Financial and material support should also be acknowledged. Groups of persons who have contributed materially to the paper but whose contributions do not justify authorship may be listed under a heading such as "clinical investigators" or "participating investigators," and their function or contribution should be described—for example, "served as scientific advisors," "critically reviewed the study proposal," "collected data," or "provided and cared for study patients."

Because readers may infer their endorsement of the data and conclusions, all persons must give written permission to be acknowledged."

> The Vancouver rules set forth three key conditions for coauthorship:
>
> 1) substantial contributions to conception and design, OR acquisition of data, OR analysis and interpretation of data,
> 2) drafting the article OR revising it critically for important intellectual content; and
> 3) final approval of the version to be published.
>
> All three criteria must be met.

Table 1: The Vancouver rules' main criteria for coauthorship

As shown by table 1, all three criteria must be met, but such that it is sufficient that one of the alternatives under 1 and 2 respectively have been met. This means that all the authors must have been involved in the intellectual process writing a scientific publication involves, see condition 2, and "the additional requirement" that all authors must have participated sufficiently in the work in order publicly to assume responsibility for suitable *parts* of the content of the publication. It must be underlined that these criteria have been under development. In Annex 2, the Commission has included a table showing how the key criteria looked at various points in time.

As can also be seen, the Vancouver rules differentiate between authors and contributors. Contributors who do not qualify as an author, by not meeting all three conditions, shall be stated and acknowledged in a separate section, "acknowledgement". Examples of such contributors may be a person who assists solely in data collection, part analyses, technical help, writing assistance or more general support.

3.5.3   The relation of medical research to the Vancouver rules

The Vancouver rules do not legally represent any form of mandatory legislation. The criteria are in their nature guidelines which hold authority arising by the degree of compliance of the principles that take place in practice.

However, several of the publications referred to above show that the Vancouver rules to a varying degree are known among medical researchers, and that they also in a varying degree are accepted and practiced by the researchers who are familiar with the principles.

Based on the global impression the Commission has gained through its work, among other things, it seems that also within medical research in Norway, there are different perceptions of the authority of the Vancouver rules. The Commission's impression is that the principles are not (or have not been) well

known in all research communities, although most people probably have "heard about" them. The Commission's impression is also that the principles in certain research communities are practiced rather leniently.

The Commission underlines that these impressions are based on a limited material, but there is nevertheless reason to express these observations, in that it appears as obvious to the Commission that any such extensive deviating practicing – or non-practicing – of the Vancouver rules in a major part of the medical research community obviously will have to be taken into consideration when assessing whether there is a basis for criticism against the coauthors' part in the case. The Commission's impression at this point is moreover in harmony with the findings that are documented in international journals.

The Commission would in particular refer to a British study including 66 researchers which found that 76% supported criteria for authorship, but that few had any knowledge of or used available criteria. Of the five persons who could specify all three Vancouver rules, only one person knew that all three criteria are to be met.[21]

The study concluded that "there seems to be a gap between editors' criteria for authorship and researchers' practice", and that "the strategy for communicating and implementing the criteria of the International Committee of Medical Journal Editors has largely failed. New initiatives should engage researchers and meet their legitimate needs. Future guidelines should be developed collaboratively and not be imposed on researchers by editors."

Similar discrepancies between the Vancouver principles and that which is practiced in medical research communities in other countries have been documented in other articles.[22]

On the other hand, it is obvious that journals as well as research institutions must be able to practice and enforce the Vancouver rules as if they were binding, and not only guiding. That means that they may make demands that authors that publicize or work for them, follow these criteria. Thereby, the criteria will be seen as mandatory for these researchers. The management at the Radiumhospitalet, for example, has clearly stated to the Commission that the Vancouver rules apply to researchers at the institution, and that it is expected that they are followed. These requirements should be seen as a binding work instruction which the employer must be able to establish and enforce. However, it is unclear to the Commission whether this instruction has reached and is being practiced by the employees at the institution, and if so, to what degree.

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[21] [21] Bhopal R et al. The vexed question of authorship: views of researchers in a British medical faculty. BMJ 1997; 314: 1009

[22] Pignatelli B, Maisonneuve H, Chapuis F. Authorship ignorance: views of researchers in French clinical settings. J Med Ethics 2005; 31: 578–581.

3.5.4 The author responsibility

Based on the Vancouver rules and other rules for authorship, it may seem unclear what responsibility the individual author has when publishing research results. In the Commission's opinion, this responsibility must be seen in the light of general liability rules, see section 3.3.

The implication for authors is that one must assume responsibility for what one has done, or as the case may be, not done, but ought to have done. Consequently, it is not necessarily the fact that one is mentioned as an author or coauthor that is decisive for the responsibility question but first and foremost what one in actual fact has done or not done. This also means that one must accept that individual authors are responsible for different elements, even when they act jointly, provided one is able to determine what the individual person has done. When several persons prepare a publication together, it is inherent in this that it is necessary to no little degree to build on and trust what a partner or other persons involved provide in the form of information of other parts of the research work. On the other hand it is of course possible that a coauthor or others will be held responsible because circumstances existed that indicated that one should have reacted and made further investigations.

However, to be an author does not imply that accepting (co-)authorship means almost signing a contract and becoming responsible for absolutely all parts of what is stated in the publication being correct. Such an interpretation has no basis or legitimacy in real life today, nor in the current version of the Vancouver rules, cf the requirement as to responsibility for "suitable parts".

3.6 Retraction of invalid publications

Medical journals have customs for retraction of invalid publications that have been published. However, the rules for so-called retraction are neither uniform nor entirely clear, and such retraction occurs relatively seldom. In section III.B of the Vancouver group's guidelines, the guidelines for corrections, retractions and expression of concern are stated as follows:

> **"III.B. Corrections, Retractions and "Expressions of Concern"**
> Editors must assume initially that authors are reporting work based on honest observations. Nevertheless, two types of difficulty may arise.
> First, errors may be noted in published articles that require the publication of a correction or erratum of part of the work. The corrections should appear on a numbered page, be listed in the contents page, include the complete original citation, and link to the original article and vice versa if online. It is conceivable that an error could be so serious as to vitiate the entire body of the work, but this is unlikely and should be handled by editors and authors on an individual basis. Such an error should not be confused with inadequacies exposed by the emergence of new scientific information in the normal course of research. The latter require no corrections or withdrawals.

The second type of difficulty is scientific fraud. If substantial doubts arise about the honesty or integrity of work, either submitted or published, it is the editor's responsibility to ensure that the question is appropriately pursued, usually by the authors' sponsoring institution. However, it is not ordinarily the task of editors to conduct a full investigation or to make a determination; that responsibility lies with the institution where the work was done or with the funding agency. The editor should be promptly informed of the final decision, and if a fraudulent paper has been published, the journal must print a retraction. If this method of investigation does not result in a satisfactory conclusion, the editor may choose to conduct his or her own investigation. As an alternative to retraction, the editor may choose to publish an expression of concern about aspects of the conduct or integrity of the work.

The retraction or expression of concern, so labeled, should appear on a numbered page in a prominent section of the print journal as well as in the online version, be listed in the contents page, and include in its heading the title of the original article. It should not simply be a letter to the editor. Ideally, the first author should be the same in the retraction as in the article, although under certain circumstances the editor may accept retractions by other responsible persons. The text of the retraction should explain why the article is being retracted and include a full original citation reference to it.

The validity of previous work by the author of a fraudulent paper cannot be assumed. Editors may ask the author's institution to assure them of the validity of earlier work published in their journals or to retract it. If this is not done editors may choose to publish an announcement expressing concern that the validity of previously published work is uncertain."

3.7 In detail on education of researchers/training of researchers and the supervisor role

Since 1993, the national regulations called Regulations for PhD Degrees with Requirements as to an Organized Education of Researchers have formed a common basis for organized education of researchers in Norway. The organized researcher education implies that the traditional PhD in arts and sciences gradually is to be replaced by doctor degrees specific to special subjects, mandatory course teaching was introduced to make the researcher education wider, and the relationship between the PhD candidate and his/her supervisor was to be formalized through written agreements.

The universities have an overall responsibility for the education of researchers in Norway. Yet, an estimated third of the PhD candidates have their main place of work at other institutions, and to a considerable degree receive supervision by persons who are not employed at the universities. In addition to the PhD candidates' own intellectual qualities, it is the supervisors' and the research community to which the candidate is related that is of the most importance for the quality and efficiency in the education of researchers. The relation between supervisor and PhD candidate is here a crucial item.

Analyses of development in the organized education of researchers show large variations as regards adaptations to the common regulations, in its practicing, in the interpretation of the professional

requirements to a PhD degree, in attitudes to supervision and how the scope and organization of the course part is viewed. Variations are in particular related to various lingering subject-specific traditions, and tensions between the requirements as to an independent research effort and the requirements as to the supervisor's contribution in the work with the dissertation often seem to arise.[23]

To be a research recruit means essentially to complete a researcher education with the achievement of a PhD degree as the final goal. Seen this way, the PhD candidate will be in an education situation, having a role with certain similarities to the role as a student.

On the other hand, the PhD candidate has completed his/her university education at master level, and he/she also often has a certain work experience. The main part of the PhD degree education consists in fact of more or less independent research. In this respect, the PhD degree student may be compared with ordinarily engaged scientific and/or clinical staff. This tends to make the research recruit's position close to that of ordinary scientific employees regarding rights and obligations.

As a starting point it may therefore be natural to consider the recruits as students when they study and participate in courses/seminars and the like, and as employed scientific staff when they otherwise are engaged in research.

The personal responsibility of the recruits, however, must be decided concretely in relation to the individual situation. Some candidates work rather independently and appear as de facto project managers for their PhD degree project, for which they also have a considerable/the main responsibility for the planning, implementation and completion. Other recruits will often be in a far more subordinate relationship, in which typically the supervisor is also the project manager, co-researcher and holder of the day-to-day responsibility for the PhD candidates' projects from inception to end, without this excluding a personal responsibility also for the recruit.

Thus, to be a supervisor is a central task in the education of researchers. In spite of the guidelines mentioned above, there are clearer and more unambiguous rules for the role of the supervisors as regards training in good research practice in a wide sense (vocational ethics for researchers). The supervisor role and the status held by the supervisor is to a large degree based on customs in the research communities, adapted to special circumstances at the individual institution, the individual professional and research community, and not least specific agreements between and circumstances related to the individual supervisor and/or candidate relationship. In the booklet PhD guidelines, an idea booklet prepared by three Danish medical researchers, the following is stated on page 8:

> "The purpose of the guide is, in a master study situation, to inspire and comment on the PhD candidates'
> personal effort and the work emerging thereof. In addition, as a supervisor one is to act as a personal

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[23] Kyvik S. Forskerutdanning *[Education of Researchers]*. In Magnus Gulbrandsen M, Smeby JS (ed.). Forskning ved universitetene *[Research at the Universities]*. Oslo: Cappelen Akademisk forlag, 2005.

support. The aim of the guide is not primarily to disseminate knowledge relating to methods, but rather to be a catalyst for the candidates' development as a researcher . … Supervising is a process, in which by a combination of inductive and deductive pedagogy shall help the PhD candidate to acknowledge problems and find solutions to them."[24]

These general remarks seem to have some relevance also in a Norwegian context.

The booklet also states that a supervisor is not necessarily the same as a project manager. The supervisor may of course be the project manager, but such a double function is not automatic.

Nor is it automatically so that as a supervisor, one is also to be a coauthor or last author on the candidate's publications, although especially within the medical community a certain tradition for this has developed. The supervisor must like everybody else meet the criteria for authorship to be listed as an author.

Thus the supervisor will basically appear as an advisor and conversation partner, unless more committing responsibilities and rights follow from other circumstances, e.g. that the supervisor also is placed in the line above the candidate and is the closest superior of the latter.

Gradually it has become customary that when admitted to a PhD degree program, the candidate enters into a contract of professional direction in the PhD degree education. On the other hand, these contracts are often so vague that they provide little guidance beyond normal customs. In the absence of clear agreements and rules, the supervisor's responsibilities and duties must therefore be determined specifically.

According to the PhD degree program at the Medical Faculty, University of Oslo (adopted June 14, 2005), for example, the following rules apply:

Section 8 Supervision

The PhD candidate and the supervisor shall be in regular contact. If the PhD candidate has several supervisors, a main supervisor with the primary responsibility for the professional follow-up of the PhD candidate shall be appointed.

At least one of the supervisors must be employed by the faculty at which the PhD degree candidate is admitted or at another entity at the university approved by the faculty. All supervisors shall have a PhD degree or corresponding professional competence. Both the (main) supervisor and PhD candidate are obligated to report in accordance with the regulations stipulated by the faculty.

The supervisor is, in consultation with the institution, responsible for arranging for the PhD degree candidate's regular participation in an active research environment. For PhD degree candidates being associated to another institution, an agreement shall be entered into between the institution awarding the degree and the cooperating institution which shall regulate the working conditions which shall include ensuring the PhD degree candidate's participation in an active research environment.

The frequency of the formalized supervisor contact (individual supervision and group supervision) shall be set forth in the agreement.

At least one supervisor is to be associated to the medical faculty at the University of Oslo.

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[24] Bentzen N, Hansen BL, Nexøe J (red.). Ph.D.-vejledning. Et idéhæfte [PhD guide. An ideas booklet]. Købehavn, 1999.

The supervisor shall:

- Advise on the formulation and delimitation of the subject and problem for discussion
- Discuss and consider hypotheses and methods
- Assist the candidate in finding his/her way in the relevant literature and raw data (library, archive, etc)
- Discuss the arrangement and execution of the presentation (disposition, language, documentation, etc.)
- Keep updated on the progress of the candidate's work and evaluate it in relation to the work plan
- Assist in introducing the candidate to relevant scientific environments
- Discuss results and their interpretation
- Guide the candidate in research-ethical questions related to the dissertation

The PhD degree candidate shall:

- Submit reports or drafts of parts of the dissertation to the supervisor in accordance with the PhD degree agreement
- In his/her work comply with the research ethical principles applicable to the subject area.

The parties are obliged continuously to inform one another of all matters of importance to the accomplishment of the PhD degree education. The parties are obliged to actively follow up circumstances that can cause a risk of a delayed or failing accomplishment of the PhD degree education, in order that the education as far as possible may be accomplished.

The PhD degree candidate and supervisor may, if they agree, ask the admitting body to appoint a new supervisor for the candidate.

If a PhD degree candidate or supervisor should find that the other party does not comply with his/her duties, the party alleging that there is a breach of obligations is obligated to bring this up with the other party. The candidate and supervisor shall jointly try to find a solution to the situation that has occurred.

If a PhD degree candidate or supervisor finds that the other party does not comply with his/her duties, and the parties' following discussions have not agreed on how to solve the situation, the candidate or supervisor may ask to be released from the supervisor agreement. A request to be released from the supervisor agreement shall be addressed to the medical faculty and forwarded via the basic entity. A copy of the request shall be sent to the other party by the party bringing the case. Any decision to release the PhD degree candidate and supervisor from the supervisor agreement shall be made by the medical faculty.

It lies to the medical faculty to approve a change of supervisor when the supervisor or the PhD degree candidate has asked for such a replacement.

The supervisor may not in any case step down until a new supervisor has been appointed. Accordingly, in the application form for admission of Jon Sudbø on the PhD degree program in December 2000, an agreement was entered into on professional supervision with the following main elements:

5. THE SUPERVISION RELATIONSHIP

In the professional supervision, the supervisor shall in particular:

- Give advice on the formulation and delimitation of the subject and presentation of the problem

- Discuss and evaluate hypotheses and methods
- Give assistance in getting acquainted with literature and raw data (library, archives, etc.)
- Discuss the arrangement and preparation of the presentation (disposition, language, documentation, etc.)
- Keep informed of the progress of the candidate's work and evaluate it in relation to the work plan
- Assist in introducing the candidate to relevant scientific environments
- Discuss results and their interpretation

The PhD candidate undertakes to submit reports or drafts of parts of the dissertation to the supervisor, as the case may be in connection with seminars, every ….

Both parties in the supervision relationship are entitled to regular contact and information on the progress of the work. The framework for this is to be determined by the body approving the annual progress report, cf item 4.

3.8   Retention of research material – obligation and right

3.8.1   The problem at issue

Material used in medical and health research often consists of human biological material (including bio banks), data files (including personal data registers), case notes, analyses, memos, draft manuscripts and the like. The research material is the basis for the research results. To enable a future check of whether the research results are correct and/or arrived at in a sound manner, it is often a prerequisite that the underlying research material can be examined.

In this context there can be question of, and if so, how and for how long, researchers must retain the research material (retention obligation).

Another problem is related to the retention *right* – i.e. the researcher's right to manage the research material: May researchers delete research material whenever they want to, or keep the research material for as long as they wish and do with it as they themselves find serviceable?

Some rules and guidelines on this exist, but they are unclear and fragmented and for that reason fairly unknown.

3.8.2   The retention obligation

According to good scientific practice, raw material shall basically be retained in order to ensure checks. The period of retention according to scientific practice is difficult to state. For example, this norm is

specified in the Regulations on Clinical Testing of Medicines, in which section 5-3 (2) and (3) state that it must be ensured that source data are available at the place of testing for at least fifteen years from the date of the final test report. The Nylenna Committee has also recommended the enactment of a retention obligation for all raw material for ten years after the completion of the research project (NOU 2005:1). In addition, there are relevant rules on this in the Bio Bank Act, the Personal Data Act and the Personal Health Data Filing System Act, which the Commission does not find reason to comment on in detail. There are also rules for the retention and processing of documents in the health service in, i.a., the Specialist Health Service Act, the Health Personnel Act and the Patient Case Notes Regulations. The employer may also have established internal instructions on this. Moreover, documents prepared by employed researchers at public institutions, as for example a public hospital, even if it is not practiced like that currently, may be subject to more general rules on the retention and processing of documents as for example the Archives Act. The retention obligation must of course take appropriate account of the requirements made to the processing and storing of person-identifiable information.

As an example may also be mentioned that the Research Council of Norway in its standard form contract provides that the research material shall be stored in line with good scientific practice. The prevailing contractual condition "Standard form contracts and granting letters – R&D" state among other things:

> "Unless otherwise provided by the body authorized to decide the use of the data, copies of all research-generated data, including necessary documentation, shall be transferred to the Norwegian Social Science Data Services. Such transfer shall take place as soon as possible, and no later than two years after the conclusion of the period to which the project grants apply. The data that are to be comprised by this must be agreed specifically with the Research Council. … The person responsible for the project is responsible for following relevant standards of quality, statutes and other public regulations. Where test persons/patients/clients are included in research projects, a recommendation by the regional committee for Medical Research Ethics according to the prevailing rules relating to the obligation to submit is required. The project-responsible person is responsible for the recommendation being complied with. … The project-responsible person shall file the final test report in an adequate way for a minimum of ten years after the completion of the project. The project-responsible person is under a duty to ensure that the data are stored in such a way that they will also be taken care of and be available should the project-responsible person cease to have such a responsibility. The project-responsible person is obligated to follow recognized quality norms when collecting and filing data. Any breach of obligations relating to the reporting and filing will be considered as a fundamental breach and thus give the Research Council reason to cancel the contract, cf section 12.2. …"

Since 1995, the Research Council has had an agreement with the Norwegian Social Science Data Services on the filing of research data relating to medical and health research. For the year 2000 this included that "The project-responsible person shall file the final test report and project data in an adequate way for a minimum of ten years after the conclusion of the project. The project responsible-person is under a duty to

ensure that the data are kept in such a way that they will also be taken care of and be available if the project-responsible person is dissolved" (read: the institution).

3.8.3 Right of retention and management

As regards the question of the right of management in more general terms, reference is made to the fact that research material, and in particular the use and retention of human biological material and personal data, collected by or under the auspices of a research institution, will, as the obvious main rule, be subject to the institution's overall responsibility, and thus also to the institution's right of management. This type of sensitive material is not the private property of an employed researcher.[25] This must now be fairly clear, although a district court decision from 1999[26] may be cited in support of the opposite solution.[27]

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[25] NOU *[Norwegian Official Reports]* 2005:1: <u>God forskning – better health</u> *[Good Research – Better Health].* Act relating to medical and health research that involves humans, human biological material and health data (the Health Research Act)

[26] Rettens Gang *[a Norwegian law reporter of court decisions in the first instance and courts of appeal]* 2000, p. 1010.

[27] Simonsen S, Nylenna M. <u>Helseforskningsrett</u> *[Health Research Law]* Oslo: Gyldendal, 2005

4.   Clarification of the facts

4.1   The cause – the Lancet article

In December 2005, Camilla Stoltenberg, Division Director at the Norwegian Institute of Public Health, read an article published by a group of Norwegian and foreign researchers in the internationally highly respected journal The Lancet in October the same year:

> Sudbø J, Lee JJ, Lippman SM, Mork J, Sagen S, Flatner N, Ristimaki A, Sudbø A, Mao
> W, Evensen JF, Reith A, Dannenberg AJ. Non-steroidal anti-inflammatory drugs and the
> risk of oral cancer: a nested case-control study. The Lancet. 2005 Oct 15-21;366:1359-66.

While reading the article, Stoltenberg became aware of certain cited factors which she was not able to see agreed with actual facts. This was discussed with other researchers at the Institute of Public Health. The Cancer Registry, which had been stated as supplier of the cancer cases, was also contacted. The Cancer Registry further contacted Professor Lars Vatten, MD, of the Norwegian University of Science and Technology (NTNU). Vatten is among other things a member of the management group for Cohort of Norway (CONOR), to which a reference was also made in the article. CONOR is both the designation of a collection of health data and blood samples, and a collaboration between the Public Health Institute and the universities relating to regional health studies. In addition, Vatten is involved in HUNT (the Nord-Trøndelag health study). Vatten read the article and reacted to several of the discrepancies in relation to actual facts. He brought this up in an email which was sent to the first author, Jon Sudbø, on January 5, 2006. On January 10, a meeting was held with Jon Sudbø and Albrecht Reith at the Cancer Registry, at which Vatten among other persons was present. On January 12, Jon Sudbø admitted to his superiors at the Radiumhospitalet that he had fabricated the data file on which the research results presented in the Lancet article was based. This means that the alleged patients from whom the analyzed data originated, were fictitious. Jon Sudbø has later on confirmed this to the Commission. Jon Sudbø has also stated that there are deficiencies in two other articles:

> Sudbø J, Samuelsson R, Risberg B, Heistein S, Nyhus C, Samuelsson M, Puntervold R, Sigstad E,
> Davidson B, Reith A, Berner A. Risk markers of oral cancer in clinically normal mucosa as an aid in
> smoking cessation counseling. J Clin Oncol. 2005 Mar 20;23:1927-33

> Sudbø J, Lippman SM, Lee JJ, Mao L, Kildal W, Sudbø A, Sagen S, Bryne M, El-Naggar A, Risberg B,
> Evensen JF, Reith A. The influence of resection and aneuploidy on mortality in oral leukoplakia. N Engl J
> Med. 2004 Apr 1;350:1405-13.

The Lancet article has subsequently been retracted. Journal editors have issued so-called expressions of concern regarding the articles in Journal of Clinical Oncology 2005 and New England Journal of Medicine 2004, as well as two articles by Sudbø et al published in the same journals in 2002 and 2001 respectively.[28]

On this background, the Commission found reason to investigate the entire scientific activity and production of Jon Sudbø. The Commission also found reason to investigate the role of all the coauthors and other players.

The Commission's primary task has been to map the material which forms the basis for the publications. Important questions have been:

- Are the patients who allegedly have been studied real or fictitious?
- Have patient data been manipulated?
- Do serious methodological flaws exist?
- Are there evident and serious flaws in the research reporting?
- Are there other serious breaches of scientific practice/good research practice?

In line with these points of departure, the Commission started its work by mapping the data basis which is the foundation of Jon Sudbø's first big scientific project, the PhD degree project. The background for this choice is that the raw material collected in connection with the PhD degree project, as well as the results thereof, has been used in and forms the basis also for Sudbø's subsequent research.

Jon Sudbø has been presented with the draft report, i.e. what corresponds to Chapter 4 through section 7.2.1 of the prevailing report on two occasions. In a letter of May 30, 2006, he submitted a series of comments to the first draft report which the Commission has compared with information which otherwise has come to light during the investigation. The Commission has corrected the draft on points where one found a factual basis for taking account of Sudbø's comments. However, the Commission would emphasize that these comments have not entailed any significant changes in the Commission's assessment of the validity of Sudbø's research work. A substantial part of Sudbø's objections are linked to the relationship between him and his supervisor, Reith, in that Sudbø alleges that the supervisor had a much more key role in the research project than the impression the Commission has got through the investigation otherwise. The Commission

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[28] Horton R. Retraction—Non-steroidal anti-inflammatory drugs and the risk of oral cancer: a nested case-control study. The Lancet 2006; 367:382, Expression of concern for Sudbø et al., J Clin Oncol 23: 1927-1933. Journal of Clinical Oncology 2006; 24: pp. 2404; Curfman GD, Morrissey S, Drazen JM. Expression of concern: Sudbo J et al. DNA content as a prognostic marker in patients with oral leukoplakia. N Engl J Med 2001;344:1270-8 and Sudbo J et al. the influence of resection and aneuploidy on mortality in oral leukoplakia. N Engl J Med 2004;350:1405-13.

will at some places comment on diverging perceptions of the facts where this is deemed necessary. Sudbø chose not to comment on the revised draft report, sent to him subsequently.

4.2 The PhD degree project relating to oral cancer

4.2.1 Introduction

Jon Sudbø is educated as a dentist (cand. odont.) from 1985 and medical practitioner (cand. med.) from 1994 with the very best grades. In his odontology study he came best in his class. In 1993 Sudbø, in cooperation with Professor Albrecht Reith, MD, at the Radiumhospitalet applied to the Cancer Society of Norway for funding of his PhD degree project. The study concerned finding methods to predict oral cancer. Sudbø has stated that it was Albrecht Reith who took the initiative to the project at issue. This took place by Reith making contact with Associate Professor T.Ingar Leidal at the Faculty of Odontology at the University of Oslo, to ask him to recruit a candidate to a research project on oral premalignant diseases, which Reith had been planning for some time. After this first contact in February 1993, Sudbø was contacted by Leidal, who thought Sudbø might be suited for such a project. However, Sudbø had not previously worked with this problem, which he found interesting. The first contact between Sudbø and Reith took place in February or early March 1993. The Cancer Society granted Sudbø a three year stipend, with Albrecht Reith as main supervisor. Sudbø was then from 1994 paid a salary as a recruitment fellow by the Cancer Society.

4.2.2 The subject matter of the doctor degree project

Background: No methods exist to indicate which oral dysplasias may also develop into cancer.

Goal I: The project description from 1993 states that Sudbø and Reith wanted to study the malignant transformation potential through a historical prospective study of leukoplakia materials, in order to map out any structural DNA changes in dysplasias, and compare these findings with the persons who later on developed cancer. They also wanted to start a prospective study. The aim was to be able to say something about the prognosis of premalignant conditions. Image analysis methods were also to be tested. The material for the historical prospective study was according to the project description already available, whereas the material for the prospective study was to be obtained by scraping of suspicious mucous areas.

Goal II: In addition they wanted to study manifest cancers. The hope was to map out the DNA changes which identify cancers with a good and poor prognosis. The project description states that the material had

been collected. Sudbø has stated that he is unable to understand that the project description states that the material had already been collected. He alleges that there is no doubt that this material was collected after he started as a recruitment fellow. The Commission finds it predominantly likely that Sudbø, in a project which he had just heard about a few weeks before, did not at this point in time have the opportunity to collect the material in advance. Sudbø assumes that the project description is formulated to reflect that the project had been prepared by Reith. The Commission finds, and this is supported by Reith, that the formulation in the project description reflects the fact that Reith through his contacts had knowledge about the material that had been collected at Gade's Institute (and possibly also the material from the faculty of odontology), to which the Commission will revert later on.

4.2.3   The organization of the PhD project

Jon Sudbø's PhD project was carried out during the period 1993-2001. The PhD project has a rather unclear, but at that time hardly unusual, basis and organizational structure, which the Commission has seen reason to try to clarify and to which it will relate some remarks. The organization of the PhD project and the formalities in that connection, is, i.a., of great importance to the everyday as well as the overall responsibility for the planning, implementation and completion (reporting) of the project.

Jon Sudbø first received stipend funds from the Cancer Society from January 1st 1994 till December 31st 1996. In 1996 he was in addition granted a finalizing year. During this period, the Cancer Society formally was his employer. As from August 1, 1996 until January 19, 1998, Sudbø obtained a leave of absence to complete his internship. In addition, he ran his own private dentist practice at Årvoll Dentist Center sharing an office with some colleagues.

During the two first years, Sudbø did not have a formal employment relationship at Radiumhospitalet. But his main supervisor – Albrecht Reith – was employed as a researcher at the department of pathology, division for digital pathology. According to the usual practice when projects were financed externally, office space and the practical organization of Jon Sudbø's work were arranged at this department. From January to September 2000, Sudbø received a stipend from the cancer research institute at the Radiumhospitalet, and he was then formally temporarily employed at the Radiumhospitalet. The head of the department of pathology was, and is, Professor Jahn Nesland MD. Professor Håvard Danielsen PhD was head of section at the division for digital pathology.

Sudbø has expressed surprise that he did not have any formal employment relationship at the Radiumhospitalet the first years when he worked at the department of pathology, among other things

because Jahn M. Nesland in his capacity as departmental chief physician recommended the application to the Cancer Society. He also alleges that it was not until 2005 that he realized that Reith was not a professor at the University of Oslo.

In any case, it is a fact that Jon Sudbø and Reith performed their daily work at the Radiumhospitalet, and that the research took place there. This fact was known and accepted by the Radiumhospitalet. The Commission will also point out, as stated by Reith, that Sudbø's PhD work was a continuation of three other PhD works which Reith had supervised prior to Sudbø's project, and that these four projects, which were all supported by the Cancer Society, were concentrated on the same matter – the connection between early stages of cancer and subsequently developed cancer.

Thus, the Commission finds that the *real* association with the Radiumhospitalet appears as so strong that in reality one is dealing with an association relationship which is fully comparable with an employment relationship. Accordingly, the Commission finds that the Radiumhospitalet has had a customary daily management and instruction right in relation to Jon Sudbø during the entire PhD period, and that the research has taken place under the auspices of the Radiumhospitalet. Most of the conversations with the Commission are clearly indicative of this. It is also, for example, evident that both Gade's Institute in Bergen and the Cancer Registry related to the Radiumhospitalet as an institution, and not to Jon Sudbø as a private person. The latter would also be contrary to expectations.

The Commission thus finds that the Radiumhospitalet had the primary responsibility for Jon Sudbø's research.

It was not until November 2000 that Sudbø applied for admission to the PhD program at the University of Oslo. It was then fairly obvious that the dissertation was already as good as completed. When the management of the University of Oslo informed Sudbø of the fact that he had not been admitted to the program, he brought this up with Reith, who according to Sudbø's statement made the excuse that it was all an oversight. Sudbø has underlined that he – in spite of failing to be admitted to the PhD program – had participated "in all the mandatory research education courses" and that these were paid for by the Radiumhospitalet. The Commission has not found it serviceable to deal with this in more detail.

Sudbø was admitted to the program at the end of December of the same year, at the same time as the dissertation was submitted. Sudbø's dissertation was approved on February 20, 2001. The presentation of the thesis took place on March 9, 2001, and he was created a doctor in June, 2001. The main supervisor was Albrecht Reith. Since Reith was not a professor at the University of Oslo, Jahn Nesland, who held a professor II position at the University of Oslo, was appointed so-called contact supervisor. Nesland was the only one at the department who had such link to the University of Oslo, and he therefore held a series of such administrative positions. His position as contact supervisor was, in other words, established for formal reasons, by Nesland acting as the connecting link between the University of Oslo and Sudbø. Real supervision by Nesland was not an issue to any particular degree. Consequently, Nesland is not a coauthor of any of the publications resulting from the PhD project. The University of Oslo has thus primarily been

responsible for the approval of the dissertation and the thesis. The Cancer Society has exclusively acted as a funding source, and has not had anything to do with the organization and implementation of the project.

Conversations with the Commission clearly indicate that Jon Sudbø had a relatively free position as a fellow, a situation that is not unusual. Sudbø is described as clever, ambitious, experienced and independent compared with other fellows. Reith had a wide network which was used to obtain patient material among other things. Sudbø and Reith had a close relationship, with almost daily contact, during which professional questions seem to have been regularly discussed, although on a more general level. Reith has thus obviously been more peripheral in relation to being involved in the data material, data analyses and the like. Consequently, Reith's role appears more as an active mentor than a co-researcher, see section 3.7 above.

However, Sudbø has denied that he had such a free position. He has alleged that his work efforts "clearly were directed by Albrecht Reith" and believes that it is not correct that Reith's role can be described as a mentor role. Sudbø believes he can support this by referring to his work effort, when he took up the position in 1994, being directed towards work with graph theory analysis on carcinoma, first prostate carcinoma and subsequently oral carcinoma. Sudbø points out that this was not at all comprised by the original project description. Sudbø has alleged that when he commented on this to Reith, he was told that he should nevertheless commence with this work, and that one could later on revert to the work related to the original project description. Thus, Sudbø is of the opinion that Reith redefined Sudbø's research project in the direction of method development. The reason for this change is allegedly that Reith had invited a French researcher to the Radiumhospitalet, Raphael Marcelpoil, as a post doc. for 12 months, after Reith having been an evaluator at Marcelpoil's presentation of his thesis in 1993. Sudbø has alleged that the work with the graph theory analysis represented the entirely dominating part of his research effort up to 1999. The Commission understands Sudbø to believe that in this work he was dependant on cooperation with others to such a degree that this does not give grounds for finding that he had a free and independent position as a research fellow, nor that Reith had a withdrawn role as supervisor. The Commission moreover understands Sudbø such that the work that was comprised by the original project description (oral premalignant diseases), and which was to comprise analyses of the material from Gade's Institute and the Faculty of Odontology at the University of Oslo, was not given priority in the period up to 1999. Reith refutes however that he is supposed to have instructed Sudbø to give priority to the work with the graph theory, and that this in all essentials was Sudbø's priority. This explanation is clearly supported by a memo written by Sudbø in 2000, in which is stated that "it appeared absolutely obvious to the undersigned [Jon Sudbø] during most of my time as a research fellow that these methods [the graph theory] sooner or later would provide results, which was the reason I continued to

work with them, although Reith and the undersigned several times discussed my priorities in this respect".

In spite of Reith's close contact with Jon Sudbø, and Sudbø's description of this relationship later on, to the Commission the project first and foremost appears as Sudbø's own project, in which he himself took care of the everyday research. It seems as if it is he alone who in fact had the full control of the research project, including the research material that came from Gade and the Faculty of Odontology, see also the letter from Sudbø to the Cancer Registry dated February 20, 1996, cited under section 4.2.7, and the letter of reply of March 22, 1996.

Accordingly, the Commission finds that Jon Sudbø *de facto* was the project manager for the PhD project, a description with which Sudbø strongly disagree. The Commission also finds that Albrecht Reith has been the supervisor and mentor for the project.

The responsibility thus seems divided with the individual person/institution having an independent responsibility. The individual person's/institution's detailed responsibilities, and what they consist of, will vary, and for that reason they must be assessed specifically.

The Commission notes that the fact that responsibilities apparently have been relatively unclear and unheeded in this case, is first and foremost an institutional management responsibility.

4.2.4   Advance assessment of the PhD project

Three independent central requirements to the advance assessment of research projects are relevant in this case.

1. Duty of secrecy

The first requirement is related to access to personal data subject to secrecy (patient data/health information). The treatment of such information is as the main rule dependent on the consent of the patient to which the information applies. There are several exceptions. One exception, which is particularly relevant, is dispensation from the duty of secrecy in connection with research. These rules have been unchanged at least for the last ten years. Subsections 1 and 2 of the Medical Practitioners Act of June 13, 1980 no 42 section 36 on "Anonymity – research" read at that time:

> "Medical practitioners may without regard to the pledge of secrecy communicate information that would otherwise be subject to secrecy concerning physical conditions or illness if individual characteristics have been deleted or changed so that the anonymity of the person concerned is protected. The Ministry may decide that information may or must be communicated for the purposes of medical research, and that this may be done without regard for the pledge of secrecy."

See otherwise the Medical Practitioners Act section 31 (the main rule on secrecy) and the corresponding provisions in the then applicable Dentists Act of June 13, 1980 no 43 sections 31 and 36, as well as the Public Administration Act sections 13 and 13d. The Public Administration Act is unamended, whereas the provisions of the Medical Practitioners Act and the Dentists Act have been repealed but reenacted in sections

21 and 29 of the Health Personnel Act. Any dispensation from the duty of secrecy must be applied for in advance. Consent was previously given by the Norwegian Board of Health, but after January 1, 2002, this authority has been delegated from the Ministry of Health and Care Services to the Directorate for Health and Social Affairs.

It is a fact that Sudbø's and Reith's oral cavity project implied the collection and processing of sensitive patient information subject to secrecy. The Commission has not found any indications that any participant's consent or dispensation from the duty of secrecy exists or that other relevant exemptions have been complied with. This means that this data processing is contrary to the then prevailing set of rules.

In this context it should be noted that research institutions and the like of course are not free to release patient information to researchers. This follows from the fact that patient information is subject to secrecy. Thus this is not only of importance to those who *obtained unauthorized access* to information subject to secrecy, but also for those who *released* patient information.

2. *Personal data protection*

Another requirement is related to the processing of personal data and creation of personal data registries in general. The main rule here is that such data processing requires a notification to or licence by the Data Inspectorate. A notification of application for a licence can also be sent via the personal data representative, if the research institution has one, in practice that will often mean the Norwegian Social Science Data Services (NSD).

It is a fact that Sudbø and Reith by collecting patient information from various institutions created a personal data registry with information that was partly highly sensitive. This data processing probably required a licence, a fact the Commission has received confirmation of from the Data Inspectorate and the NSD. There are no grounds to believe that Sudbø or Reith has applied to the Data Inspectorate or the NSD for such a licence.

3. *Ethical assessment*

A third requirement which is primarily a research-ethical requirement, but which increasingly is becoming a legal requirement, is the principle that medical or health research projects are to be assessed in advance by a regional research ethical committee (REC). This has been the system in Norway since the establishment of

the committees in 1985, but nevertheless such that the duty to submit for advance assessment gradually has been tightened up.[29]

It is a fact that Sudbø's PhD project was never submitted to REC-South (which would have been the correct body), not by Reith either. At its inception in the 1980's it seems that mainly it was only invasive studies, i.e. studies in which one exposes patients or other research participants to some form of intervention/influence, which was comprised by a research-ethical obligation to submit. The Commission therefore found reason to put the question of whether Sudbø's PhD project *ought* to have been submitted for research ethical approval.

In that connection, the Commission made an inquiry to REC-South to have clarified which rules existed in 1994 when the project was started. REC-South then stated on a general basis, without having been sent either the project description or other documents, that:

> "if the fact is that in this study, "register data was collected and linked with patient data" and that "biological samples were analyzed and telephone calls were made to patients for supplemental information on the use of tobacco where data were lacking", then there is no doubt that the study should have been submitted to REC (Regional Committee for Medical Research Ethics). For a study with registry data it would suffice to have the approval of the Data Inspectorate, but where patient data, patient journals, biological data from patients, contact with patients per telephone to obtain information, etc., were concerned, it is obvious that it should have been submitted to REC."

This fact must be seen in the light of the fact that far from all medical research projects of this type were submitted to the Regional Committee for Medical Research Ethics at that time, when probably also a justified doubt about the extent of the obligation to submit prevailed.

Failing compliance with the type of formalities we discuss here was hardly particularly unusual at that time. This is probably related to the fact that the set of rules was not well known. Improvements have probably taken place here by an increasing awareness of the set of rules among researcher, research institutions and regulatory bodies. It is likely that there is a certain connection here with discussions relating to formalities and the applicable set of rules in the wake of the BioBank Act which came into force on July 1, 2003, the Nylenna committee's report from December 2004 (NOU 2005:1), the proposed enactment of the ethical committees and a national dishonesty committee, and the consultations that have been held in connection with these proposals for legislation. However, the Data Inspectorate in 2004 discovered that only one out of 30 medical and health research projects had routines that ensured that the legislation on personal

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[29] Refer to, *i.a.,* an article by Bergsjø P. <u>Biomedisinske forskningsprosjekter hvor forsøk på mennesker inngår – hva er nå det?</u> *[Biomedical research projects of which experiments on humans form part – what would that be?]* Tidsskrift for Den norske lægeforening, 1993;113: 1443-1444.

data was complied with, and 11 out of 28 projects did not comply with the licence requirements. The obligation to delete was seldom observed, and in close to half of the projects the requirements as to consent were not fully met.[30]

The duty to submit

It may be asked who was responsible for the formalities applicable to Jon Sudbø's PhD project being in order.

In his statement to the Commission, Jon Sudbø asserted that he considered it to be his supervisor's duty and responsibility to ensure that the formalities were in order. There may be something to be said for this point of view, since it is relatively usual that the main supervisor assumes the responsibility for and/or calls attention to the need of advance approval. On the other hand, it will often be natural to involve a research fellow in such a process, particularly where an independent project in which the fellow acts as the de facto project manager, is concerned. Then it will normally be the fellow who has the best knowledge of the project, through the closer proximity to it, even if it is the main supervisor who signs the application as such and other documents related to the project.

Sudbø however points out that most of the time up to 1999 was spent on method development, and this was work that allegedly did not require any form of approval by public bodies, and that it was a project that he alleges was ordered by Reith, and which was not comprised by the original application to the Cancer Society. On this basis, Sudbø denies that he was to be considered the project manager. It is true that Sudbø admits that in parallel with this, clinical material was collected, but this took place under the auspices of his supervisor, and then it must be natural to require that the supervisor assumes the responsibility to ensure that the research project complies with the set of rules – particularly in a situation in which Sudbø by his supervisor was ordered to work with an entirely other project which he experienced as demanding.

The compliance with the set of rules to which a research activity is subject, and the principles related to good research practice, is nevertheless also to be an important part of the researcher education. In this respect, Jon Sudbø ought to have been aware of whether these factors had been complied with or not. In line with the views accounted for in section 3.7, the Commission finds that a fellow as the main rule must have a certain independent responsibility to make sure that important factors like approvals, licences and other formalities (e.g. participant's consent) are in order. This is particularly so where the fellow has such an independent position as in this case, where he probably is the only one who had the full daily control of the project. This responsibility is a personal and independent responsibility applicable regardless of the supervisor's and institution's independent responsibility.

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[30] The Data Inspectorate. Tilsyn våren 2004 *[Inspection spring 2004]*. <u>Behandling av helseopplysninger i forskning. Funn og tendenser *[Treatment of health data in research. Findings and trends]*</u>. Oslo:Datatilsynet, 2004

Here is to be noted, although the Commission has not found reason to pursue the matter, that Jon Sudbø, having completed the PhD project, as far as the Commission can see, did not take care of notification to relevant bodies such as the Regional Committee for Medical Research Ethics, the Norwegian Social Science Data Services, and the Directorate for Health and Social Affairs in connection with other research projects, including projects in which he was a supervisor. One exception exists: the so-called PROTOCOL study, see sections 4.3 and 5.3. On the other hand, Sudbø in the protocol for the stated study and other articles demonstrates a good knowledge of the formal requirements relating to this type of research in Norway, as regards participant's consent and advance assessment of research projects, among other things.

One problem, which is hardly unique in this case, seems to be a more or less unintentional and unfortunate mix of the role as a clinician (treating health staff) and researcher, in which the clinician's access to patients and patient data for research purposes is unlawfully exploited, without the formalities being in order. It is of course a positive thing that clinicians carry on research, but the combination of different roles requires openness and awareness as regards the set of rules for the respective roles in order to avoid an unfortunate mix of roles.

The main supervisor, Albrecht Reith, states that he was not aware of these rules and requirements about an ethical advance assessment and licence by the Data Inspectorate, etc. For that reason he did not see it as his task to provide for an advance assessment either. In the Commission's view, the main supervisor normally has an independent responsibility to ensure that formalities of this nature are in order before the fellow starts the research itself, either by taking care of it himself or by instructing the fellow to bring it in order. Sudbø has stated that he has difficulties in following the Commission's considerations relating to the mix of roles as a clinician and researcher.

Furthermore, it must be a responsibility for the research institutions to ensure that research projects are assessed in advance and otherwise satisfy other formal requirements. However, the Commission has got the impression that the institutions , neither the Radiumhospitalet nor the University of Oslo, have seen it as their task to check that research projects are in fact initiated and implemented in accordance with statutes, regulations or work instructions. The Commission has got the impression that the education as well as the practicing of this has not been as good as it should be at the Radiumhospitalet.

On the other hand, the Radiumhospitalet in the last 10-20 years has issued increasingly better instructions and the like as regards research on patients and patient material, i.a. with special intranet sites and a support office for clinical research (www.klinforsk.no). Nevertheless, it appears to the Commission that there has been a lack of efficient routines and internal control at the institution which could have contributed to ensuring that statutes and work instructions in fact were known among the employees and were complied with. In other words, the problem was the implementation and practicing itself of external

regulations and internal instructions. Sudbø's PhD project was never submitted to the Radiumhospitalet's Protocol Committee, for example. The guidelines from 1998 state that the Protocol Committee "shall evaluate all types of clinical research projects wanted to be performed at the Radiumhospitalet and which in one way or another involve patients or patient material. The Protocol Committee may also on its own initiative bring up cases which have not been submitted to the Committee if it may seem as if it should have been submitted." No one seems to have seen it as their responsibility to take care of any submission to the Protocol Committee.

Reference is further made to the fact that the research fellow was not required to submit documentation that the formalities were in order when he presented his thesis in 2001. It ought to be a simple and not very burdensome matter to require the submission of a protocol from the Regional Committee for Medical Research Ethics, licence from the Data Inspectorate, dispensation from the duty of secrecy, etc., together with a statement that research-ethical rules and guidelines have been complied with. The Commission has not found any reference to such rules in the PhD regulations or research fellow contract.

Although an advance assessment had not necessarily prevented other breaches of good research practice, such a review would nevertheless, in the Commission's view, have been quality assuring and awareness raising. The lack of advance assessment increases the risk of breach of the patients' integrity and is a threat to the population's trust in research. Furthermore, it is difficult for institutions to safeguard completely against dishonest researchers sidestepping the system and omitting to comply with formal rules and instructions. The system must necessarily be based on a certain degree of trust.

In spite of the Commission's limited basic material, it is nevertheless evident to the Commission that such obvious effects are linked to the institutional culture and system at the time as regards the institution's and employed researchers' attitude to the formalities, as for example the protection of personal data, that one must be able to characterize this as a system failure. The Commission has been informed of which measures are now prevailing, and has got the impression that this is a "problem area" which the management at the Rikshospitalet-Radiumhospitalet MC takes seriously. It seems as if this is an ongoing area of concentration with reinforcement and implementation of several good measures.

4.2.5   Reporting and publication of the PhD work

The PhD project can be divided in two.

The first part was carried out partly in cooperation with a post.doc. from France. This part comprises three scientific publications from 2000 which can be linked to method development and image analyses of tissue architecture based on raw material originating from Germany. In the Commission's opinion, these studies are of a less sensational nature from a scientific point of view than the main part of the PhD project. However, as accounted for in section 4.2.3, Sudbø disagrees entirely in these works having a less essential role, even if

there has been less attention surrounding these studies than the main part of the PhD project. Sudbø underlines that these studies in terms of time and work represented the main part of his PhD work, and that they are published in recognized journals. The Commission has evaluated the articles and has not found indications of errors or deficiencies related to them.

The main part of the PhD project comprises three articles published in New England Journal of Medicine from 2001 (NEJM 2001), Journal of Pathology from 2001 (J Pathol 2001) and Journal of Oral Oncology 2001 (Oncology 2001):

Sudbø J, Kildal W, Risberg B, Koppang HS, Danielsen HE, Reith A. DNA content as a prognostic marker in patients with oral leukoplakia. N Engl J Med. 2001 Apr 26;344: 1270-8.)

Sudbø J, Bryne M, Johannessen AC, Kildal W, Danielsen HE, Reith A. Comparison of histological grading and large-scale genomic status (DNA ploidy) as prognostic tools in oral dysplasia. J Pathol. 2001 Jul;194:303-10.

Sudbø J, Ried T, Bryne M, Kildal W, Danielsen H, Reith A.
Abnormal DNA content predicts the occurrence of carcinomas in non-dysplastic oral white patches. Oral Oncol. 2001 Oct;37:558-65.

The articles are based on the same patient material and must be seen in conjunction. The patient material was probably obtained by Sudbø and Reith in 1995-1996 and linked with Cancer Registry data for supplementary information in 1996. The data material and samples were thereupon probably analyzed in 1999. The publication and the dissertation were published in 2001.

This patient material and the findings presented in the publications mentioned are at the very center of Sudbø's subsequent scientific career and the series of subsequent publications.

The six articles were then collected in the PhD dissertation itself, which also contains an independent compilation and explanation of the PhD project. Thus it is the three latter articles together with the dissertation itself and the patient material these are based on, which have been the subject of thorough investigations that will be described below. The starting point is the dissertation itself.

4.2.6   The patient material – an overview

It appears from figure 5 at page 40 in the PhD dissertation, included as figure 1 in this report, that Sudbø started using human biological material (paraffin blocks with biopsy samples, tissue specimens, etc.) and person-identifiable data from 263 patients (cases). In essential, the patient material, according to New England Journal of Medicine 2001 and J Pathol 2001 as well as statements to the Commission, was obtained in the following way:

- Patient material from Pathological Laboratory, the Faculty of Odontology, the University of Oslo (hereinafter: the Odontology)

- Patient material from the Department of Pathology ("Gade's Institute"), the Faculty of Odontology, the University of Bergen (hereinafter: Gade)

- Patient data from Gade and the Odontology were thereafter sent collectively to the Cancer Registry for linkage, i.e. a supplement of further data.

The Commission, on its part, has collected extensive documentation in the form of original and processed data files from various persons and instances, as well as statements before the Commission. The Commission has also made its own comparison of different data files in order to trace patients, tissue samples, check mutual linkage, etc., as part of its own internal control. The Cancer Registry has made similar investigations. The Commission was given access to the Cancer Registry's very thorough investigations. By this, the Commission has clarified how the patient material for the PhD work was obtained, and how it originally looked, including which patients were included in the study and their disease history.

The Commission then compared a series of figures and facts presented in the publications to figures and facts in the Commission's possession.

In the following, the Commission will point out along the way the errors, deficiencies and discrepancies discovered in the three mentioned articles and the dissertation itself, including the raw material on which these publications are stated to be based upon.

Figure 1 is taken from Jon Sudbø's PhD dissertation and shows, among other things, the inclusion process. An almost corresponding figure is included in New England Journal of Medicine 2001.

ORAL PREMALIGNANT LESIONS

263 cases with red or white patches originally classified as epithelial dysplasia

21 cases of erythroplakias excluded from the study for prognostic reasons

242 cases with white patches originally classified as epithelial dysplasia

46 cases with non-dysplastic lesions

Paper III (1 outlier excluded)

217 cases included in **Paper II** for assessment of inter-observer agreement in grading of oral epithelial dysplasias

36 cases with associated carcinomas

10 cases without ample amounts of tissue

Study subjects for the survival analysis in **Paper I**

150 cases with dysplastic white patches

Survival analysis

105 cases with diploid lesions

25 cases with aneuploid lesions

20 cases with tetraploid lesions

2 cases changed from diploid to

103 cases with diploid lesions

27 cases with aneuploid lesions

3 cases of squamous cell carcinoma

21 cases of squamous cell carcinoma
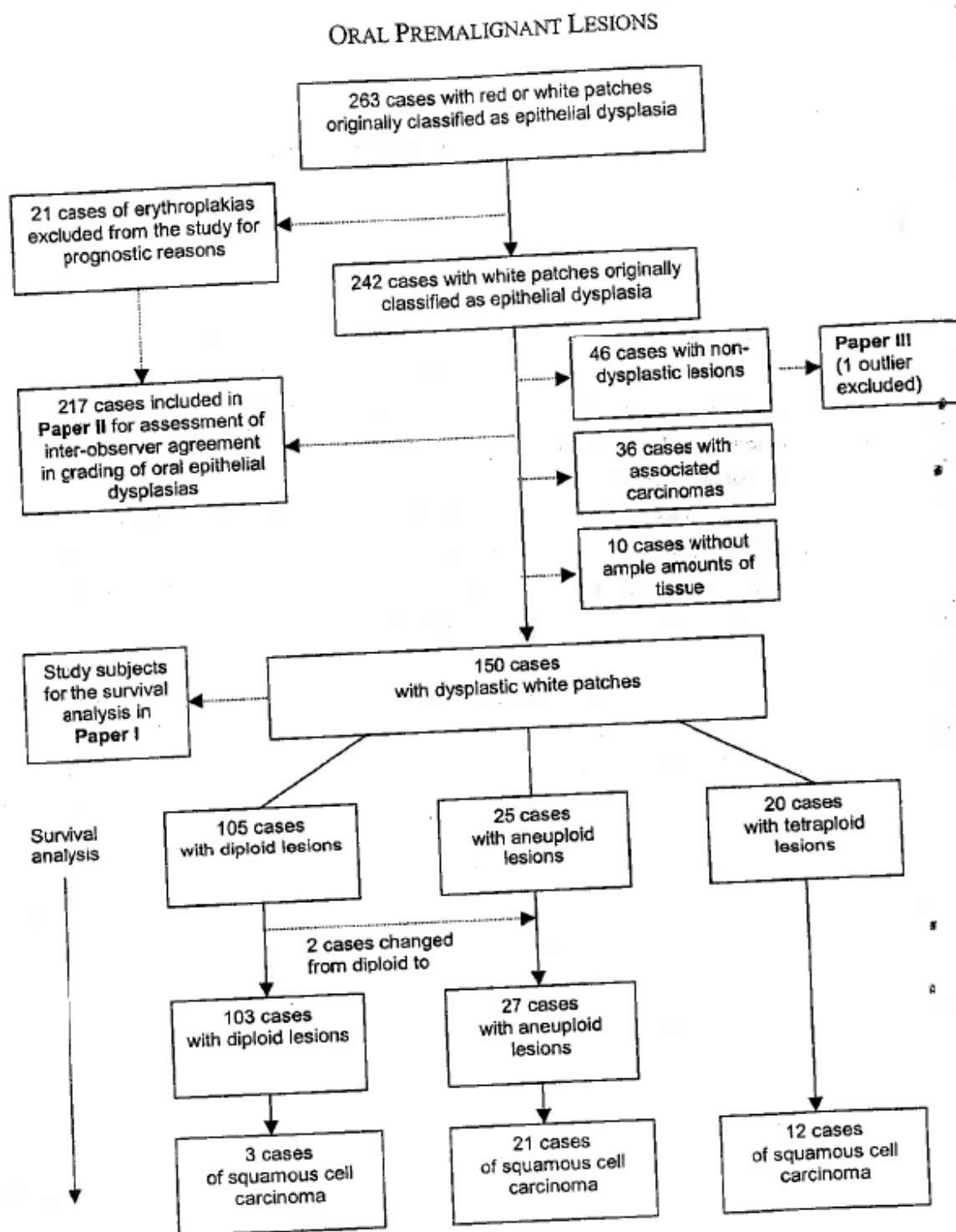
12 cases of squamous cell carcinoma

Figure 5
A flow chart showing the clinical material of oral premalignant lesions, and its use in Papers I-III. Forty-six cases originally included in Paper III, but one case was excluded, as it was regarded as an outlier because it was histolog classified as colonic mucosa and integument by 2 observers.

4.2.7 Representation of the raw material in the publications

The total number

The dissertation states that originally one had access to material from 263 persons collected during the period 1976-1995. According to the articles and the dissertation, this material originated exclusively from archives at the Odontology and Gade.

This means that Sudbø originally had access to human biological material and personal data from altogether 263 different persons. The number of samples and tissue blocks and the like was much higher, however, because normally one has several samples and blocks from the same patient. Oncology 2001 refers to all 263 patients because it includes both leukoplakias (white patches in the oral cavity) and erythroplakias (red patches in the oral cavity). In New England Journal of Medicine 2001, this starting point is reduced to 242 (263-21) because patients with erythroplakias were excluded initially. In J Pathol 2001 the starting point is 196 (263-(21+46)), although this number is 217 in the dissertation. According to Sudbø, however, it was only for 196 persons that ploidy classification existed and which for that reason were treated in J Pathol 2001. Below, the Commission will try to identify when and from where these patients come and the qualities they possess.

The time interval

In New England Journal of Medicine 2001 it is stated that the material originates from the period 1982 to 1995, whereas in the two other articles and in the dissertation it is stated to originate from 1976 to 1995. The correct time interval is unclear. According to the Cancer Registry it is supposed to be 1976 to 1995. In a letter from February 1, 1996 from Sudbø and Reith to Gisle Bang at Gade, it is stated that the material from the Odontology originates from 1984-89. In an undated letter, probably from the beginning of 1996, from Bang to Reith and Sudbø reference is made to the Gade material originating from 1981-95. In a letter from Sudbø to the Cancer Registry dated February 20, 1996, it is referred to the patients having been diagnosed 1984 up to 1995. It is surprising to the Commission that there is no conformity between the years stated. Nor is there any conformity between the stated period of time for collection in New England Journal of Medicine 2001 and the other articles.

Sudbø has denied to the Commission that there are discrepancies and "obvious errors" in the various datings, and has alleged that the total material is from 1976-1995, and that it is probable that some blocks taken before 1982 have been excluded because prior to 1982 it was not usual to use buffered formalin at the fixation of tissue blocks, which are less suited to hydrolysis and Feulgen coloring.

The ambiguities existing in relation to the time intervals are however, in the Commission's opinion, inaccuracies of such a nature that they are apt to impair the reliability of the publications.

The material from the Odontology

Sudbø states that via Reith he got access to patient material from the Odontology represented by Hanna S. Koppang. Reith has stated that he, as far as he can recall, asked Sudbø to contact professor Kjærheim at the Odontology who is supposed to have advised Sudbø to contact Koppang. According to his statement, Reith did not collect any material from the Odontology. It is somewhat unclear how many patients are involved. Sudbø states that the material was sent in several turns, and comprised patients with leukoplakias as well as erythroplakias. The Commission has not found original copies of cover letters or data lists from the Odontology which can document how many and which patients are involved.

Nor can the Commission see that any participant's consent or dispensation from the duty of secrecy exist for the delivery of the patient data, although this was a requirement for delivery of patient information subject to secrecy, see section 4.2.4. The delivery thus appears to be contrary to the set of rules enforced at that time. The University of Oslo has not been given the opportunity to comment on this fact, see sections 2.4.6 and 7.3.2.

Hanna S. Koppang, who was responsible for the pathology at the Odontology, cannot remember to have delivered material to Sudbø during the relevant period either. But the Commission can for that reason not conclude that the material was *not* delivered to Sudbø or Reith, since Koppang's statement is marked by failing recollection from this period.

Sudbø has asserted that it is surprising that professor Koppang allegedly does not remember to have delivered clinical material to Reith and Sudbø. He has stated that in the offices of Reith and Puntervold there is supposed to be lists sent from professor Koppang which allegedly shall show the number of patients, the number of lesions per patient, grading of dysplasias, transferred to *carcinoma in situ* as well as the time when these were sent. According to Sudbø, Koppang sent leukoplakias in several turns, then erythroplakias, although in a far smaller number, since these are far more rare than leukoplakias. Sudbø also alleges that he ("we") has sent the total biopsy specimens from Gade to Koppang for classification/grading of dysplasias, and that lists of this are supposed to exist with Reith or Puntervold. The Commission has submitted this information from Sudbø to Ruth Puntervold and Reith, asking whether they have seen or have had access to files, cover letters and the like which can shed light on what Sudbø has received from the Odontology and Hanna S. Koppang. Puntervold states that she received the blocks with marking of block id., and that this was the basis for the journal L 34 which was established for the preparation of ploidy samples from the Odontology. Apart from this, she has no knowledge of Sudbø's contact with the Odontology. Reith says that he now cannot find lists,

cover letters or the like. However, it should be noted that Koppang has provided the Commission with access to a list which shows that some patient samples were delivered to Sudbø in 2001. In this respect, the material from the Odontology which allegedly forms the basis for the PhD work, appears as being difficult to check.

However, the letter from Sudbø and Reith to professor Bang at Gade dated February 1st, 1996, states that Sudbø and Reith has from "… Odontology institute for pathology, the University of Oslo, been lent 83 biopsies from 63 individuals, taken in the period 1984-89. About half of these are biopsies sent by dentists from Eastern Norway, Middle Norway and North Norway. The other half is biopsies taken at the Clinic for Oral Surgery and Oral Medicine at the Faculty of Odontology, the University of Oslo …".

Moreover, Sudbø has stated to the Commission that 63 persons seem to tally. The figure 63 persons from the Odontology seems also to be in accordance with the Commission's and the Cancer Registry's own investigations. A list that the Cancer Registry made in 2006 after having themselves obtained and entered personal identification numbers for the patients who had names and dates of birth only, shows 132 observations divided on 62 different persons, which means that 70 of the observations are duplicates.[31]

Accordingly, the Commission finds that Sudbø originally and basically had access to patient data and material from around 63 different persons from the Odontology in Oslo.

The Cancer Registry's linkage

In order to have the collected patient data checked and supplemented, they were sent to the Cancer Registry for linkage. A letter of February 20, 1996 from Jon Sudbø to Chief Physician Frøydis Langmark at the Cancer Registry states:

> "I refer to our telephone conversation some weeks ago in which we discussed the possibility for a review of the Cancer Registry's data base as part of a retrospective prognostic study of premalignant changes in the oral mucosa. Regrettably, our data will arrive later than indicated to you. In the meantime, we have collected data relating to persons with the diagnosis oral dysplasia also from Haukeland hospital, and the collection and systematization of these data have taken some time.
>
> As stated, this takes place at the Department of Pathology, section for image analyses, at the Norwegian Radium Hospital, and I am currently working with a project aiming at defining morphological characteristics at premalignant conditions (dysplasia) in oral mucosa. The study is funded over three years by the Norwegian Cancer Society. Supervisor for the project is Professor Albrecht Reith, MD, Department of Pathology, the Norwegian Radium Hospital.
>
> Enclosed is a transcript of the relevant patients. This represents a collection of patients from the entire country, diagnosed from 1984 up to 1995. Personal data are given as far as they are known. I also enclose a disk with the relevant data. Could you forward this to the relevant person."

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[31] List: "Oslo_030606.xls"

The annexed list of patients comprises data from 226 patients. The Cancer Registry has referring letters and similar documentation related to the relevant patients. The Cancer Registry's subsequent investigations in 2006 show that 63 out of 226 persons originated from the Odontology. This number of patients is then in accordance with the number in the aforementioned letter from Sudbø and Reith dated February 1, 1996. The remainder (163 out of 226) originates from Gade's Institute (see below).

However, the Cancer Registry has pointed out that the persons who subsequently have been proven to originate from the Odontology did not have personal identification numbers, and that they for that reason were not linked. The Cancer Registry is certain that the only patients that were linked and returned to Sudbø were *160 persons*, who all originated from Gade (3 persons were excluded, and of these two were duplicates and one had adenoma in the stomach). Thus Sudbø received linked data for 160 patients from the Cancer Registry on March 22, 1996.

Sudbø has maintained that he was not aware that the 63 patients from the Odontology were not linked. On the other hand, Sudbø states that with the help from Reith and a laboratory technician at the Radiumhospitalet, Ruth Puntervold, he got access to the patients' full personal identification numbers from the Population Registry, and thereby was able to run a link with Cancer Registry data. Puntervold denies that she has assisted Sudbø in this. Also Reith denies that he has helped Sudbø to provide information from the Population Registry or assisted him with linkage. The Commission thus finds it quite unlikely that the patients from the Odontology had person identification numbers and that they were linked with the Cancer Registry data. In light of the information from both Puntervold and the Cancer Registry on this point, the Commission finds no grounds on which to accept Sudbø's allegations regarding the actual facts.

The cause of death registry has not registered any inquiries from Reith or Sudbø. At that time, Sudbø did not have a clinical position at the Radiumhospitalet, and thus not a free access to the hospital's case notes. Consequently, the Commission finds it to be not very likely that inadequate sets of data were supplemented in this way.

The Commission thus finds that Sudbø probably had access to human biological material and referring letter information on insufficient patient data (patient data not linked with Cancer Registry data in order to exclude coincidental and previous cancer) from 63 individuals from the Odontology. This means that all the patients from the Odontology ought to have been excluded from the study initially, and that the original number of patients should have been 63 persons less than what was stated in the dissertation and Oncology 2001.

Sudbø's assertions that the Commission is wrong on this point must be rejected, since the Commission does not find it likely that these 63 persons were linked with Cancer Registry data as alleged by Sudbø. It is also a fact that the so-called Sudbø8 file which formed the basis for New England Journal of Medicine 2004 (and thus in all essentials also New England Journal of Medicine 2001), did not comprise any persons from the material from the Odontology.

The linkage of patient data with Cancer Registry data for research purposes presupposed then, as now, that a participant consent or dispensation from the duty of secrecy existed, see section 4.2.4 and the Cancer Registry's framework licence dated December 9, 1985, cf item 4.3 of the licence. This was not so.

The Cancer Registry was notified that the Commission was considering expressing a certain criticism on this basis, and made use of its right to comment on an earlier draft report, section 7.3.4. In a letter to the Commission of June 2, 2006, the Cancer Registry submitted that the criticism in the draft is based on an erroneous perception of the Cancer Registry's different roles. The Cancer Registry alleges that they understood Jon Sudbø's request of February 20, 1996 as a routine request for follow-up data for patients at the Radiumhospitalet, Department of Pathology. The Cancer Registry alleges that delivery of the required data did not take place as "data supplier or partner in a research project, but as part of the registry role relating to the safeguarding of data completeness and follow-up of patients", focusing on the central problems which normally are involved in such a follow-up. It is alleged that the data exchange that takes place between the individual hospital and its departments and the Cancer Registry in relation to quality assurance, entails that the Cancer Registry contributes to the diagnosis and treatment departments' quality control of "their" patient material, at the same time as this normally leads to a quality increase of the Cancer Registry's main data base. The Cancer Registry alleges that this mutual quality control is "… a most central part of our registry function", and that this "activity has been ongoing on a routine basis for all years, and that it is considered to be covered by the previous licence as well as the current regulations".

Further, the Cancer Registry submitted that the draft criticism seems to be based on the assumption that the Cancer Registry should have understood that Sudbø applied for data for a *research project*. If so, this is a basis for criticism that the Cancer Registry considers to be unreasonable. The Cancer Registry in this connection refers to the fact that Sudbø's approach was accompanied by a detailed list of patients, that the cover letter stated that Sudbø's project took place at the Radiumhospitalet, Department of Pathology, and that data had also been obtained from Haukeland Hospital. The Cancer Registry submits that in light of all facts relating to Sudbø's approach in 1996 there was no reason to believe anything else than that the patient list concerned the Radiumhospitalet's own and called-in biopsy preparations, and that the request was made as a request for follow-up data – a type of requests that the Cancer Registry receives and deals with continuously. It was not until 2006, when this case had come to light, that the Cancer Registry realized that the material which Sudbø in the request from 1996 wished to have quality-assured, did not concern the Radiumhospitalet patients, but material from Gade's Institute and the Institute of Odontology's department of pathology. The

Cancer Registry furthermore submits that if the facts had been correctly stated in 1996 Sudbø would not have been given Cancer Registry data. The Cancer Registry in this connection refers to the fact that Sudbø's request in 1994 was refused exactly because he wanted follow-up on other institutions' patient material, and also wanted personal access to the Cancer Registry's data base.

The Commission has considered the remarks made by the Cancer Registry and compared them with the information provided in Jon Sudbø's letter of February 20, 1996 to the Cancer Registry, quoted above, and other information. On that basis, the Commission finds that it appears from the letter that it is a question of a research project, something which the Cancer Registry ought to have understood. The handing over of the data thus appears to be contrary to the licence conditions, se otherwise section 7.3.4.

The material from Gade

In the aforementioned letter of February 1, 1996 from Sudbø and Reith to professor Bang of Gade, it is further stated:

> "… We also have an agreement with chief physician Langmark at the Cancer Registry relating to obtaining data regarding which of these persons later developed oral cancer. However, our existing material of 63 individuals is too small to be able to be used in such a retrospective study and our question to you is therefore whether you could place a corresponding material at our temporary disposition. To begin with, we are interested in personal data from individuals who have been given the diagnosis of mild, moderate or severe squamous epithelium dysplasia from the oral cavity, regardless of localization. If data from the Cancer Registry should show that the percentage that has developed oral cancer is sufficiently large, it would also be of interest to borrow the biopsies for the production of biopsy specimens for coloring and examination."

A data list with patient data for the period 1981-95 was sent in an undated letter from Bang to Reith and Sudbø. The Commission cannot see that any participant consent or dispensation from the duty of secrecy for the delivery of patient data exist, although this was a requirement when patient information subject to secrecy was to be delivered, see section 4.2.4. Thus, the handing over of the data appears to be contrary to the set of rules applicable at the time, see also section 7.3.3. The University of Bergen has not commented on the Commission's draft criticism. A letter dated May 15, 1996 from Reith to professor Andreas Myking at Gade (to whom Bang had referred Reith), states:

> "We have at our department been in contact with professor Gisle Bang of Haukeland Hospital. He has sent us data from 161 patients diagnosed with dysplasia from mucosa (see the annexed list). We have compared these data with information from the Cancer Registry. It appears from these data that approximately 50% (79/161) of the patients with the diagnosis mild, moderate or severe mucosa dysplasia within a five years' period

developed squamous cell carcinoma of the oral cavity … Our question to you is therefore whether it is possible to be sent **cut blocks and copies of referring letters** from the patients in the lists we have enclosed …"

Sudbø and Reith were sent referring letters from Gade, a fact that is confirmed in, i.a., a letter dated December 9, 1996 from Sudbø to Professor Anne Christine Johannesen at Gade. The Gade material originates from clinics spread all over Western Norway.

The patient number of 161 persons is confirmed by the Cancer Registry, which in February 1996 linked 161 persons with the Cancer Registry's data. (Two out of 163 were duplicates. One more person was according to the Cancer Registry furthermore excluded because of adenoma in the stomach.) Consequently, the list which Sudbø and Reith got from the Cancer Registry comprised linked data from 160 or 161 persons. The Cancer Registry's investigation in 2006 shows that all 161 persons who were linked originated from Gade.

The aforementioned letter dated December 9, 1996 from Sudbø to Johannesen states:

"Enclosed please find a set of copies of referring letters I have received from Professor Gisle Bang, relating to dysplasia from the oral cavity. You will also find enclosed a Microsoft Excel spreadsheet stating patient data as they are linked from referring letters to data from the Cancer Registry. What is particularly striking with the material, is the high share of dysplasia showing malignant transformation (50%). It therefore seems to be necessary to have verified the original diagnosis (mild, moderate or severe dysplasia) and exclude any CIS. You have been in contact with Professor Albrecht Reith regarding this material, and you probably know the problem from this. Thank you for your interest and will to assist in this!"

The letter of reply from Johannesen to Jon Sudbø dated May 21st, 1997 states:

"… I have looked at all the biopsy specimens from the pile I was sent. I have a series of comments which I shall try to express as clearly and briefly as possible:

- I have tried to make a list of names, P-numbers (biopsy number) and diagnosis. All this is in normal type.
- My objections are in italics.
- Some referring letters are lacking in relation to the list received. These are mentioned by name (italics) without any further text.
- At many places there is no correspondence between the diagnosis on the list received and the biopsy answer! Here biopsy answer must of course apply.
- The explanation of why the material from Bergen has such a high frequency of malignant transformation is that you have perhaps not compared the time of their dysplasia diagnosis with the time of the malignant diagnosis. For example, at several places it appears that dysplasia diagnosis lies in a resection border in a carcinoma that has already occurred. This is therefore no

malignant transformation but a dysplasia that has already had a cancer diagnosis. Several of the

patients have also previously been operated for squamous cell carcinoma.

- As regards the diagnoses, I have not changed many. I have accepted a deviation of 1 degree (mild-

moderate, moderate- serious) without having corrected the diagnosis, this because the grading to

such a relatively large extent is subject to personal assessment. Where there are obvious errors or

major deviations, I have corrected the diagnosis.

I hope this is sufficiently clear. Then I want to wish you good luck with your further work!"

It is to be noted that Johannesen's list comprises data from 144 different persons only. Reith thinks the letter from Johannesen looks "straightforward", but he wonders why the Cancer Registry only excluded two persons out of 163, whereas Johannesen apparently excluded a further 17 persons. Reith states that he cannot recall having seen/read the letter before.

The Commission asked itself the same question. The probable explanation is that the list of 163 observations, which originated from Gade, and which were linked by the Cancer Registry in the beginning of 1996, comprised two duplicates, so that the list comprised 161 persons after linkage. In a list recently prepared by the Cancer Registry there is a column called "Diagnosis on A.C. Johannesen's quality assurance list".[32] This column contains 17 blank observations (which also are persons). This probably means that Johannesen has quality assured the diagnosis of 161-17=144 persons. This agrees with the figures the Commission's own investigations have produced, i.a. through comparisons with block numbers, see below. This is also consistent with what Johannesen wrote in a letter of May 21, 1997: "There are some referring letters that are missing in relation to the list sent. These are mentioned by names (italics) without any further text."

Total number of patients: This means that Sudbø and Reith in the summer 1997 were left with a reclassified data file from Gade's Institute which was linked with the Cancer Registry data, with data from only 144 persons, i.e. 119 persons less than what is stated in the dissertation and Oncology 2001.

The Commission finds it surprising that in the communication with Gade, after the linkage with the Cancer Registry data, there is no reference to the material from the Odontology. If the 63 persons from the Odontology, which the Cancer Registry has not linked, and which for that reason should have been excluded, nevertheless are added, the data material in the best case consists of data from 144+63=207 different persons.

It should be noted that the number of *samples* (tissue blocks, biopsy specimens, etc) far exceeds this number, because there are several samples from the same person, something which is totally usual.

Sudbø has expressed that he has no qualification to comment on these figures, stating that it was Reith who was responsible for the handling of data files between Gade's Institute and the Cancer Registry. However, the letter from Johannesen was addressed to Sudbø alone, although Reith does not exclude in his

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[32] Memo from the Cancer Registry of March 7, 2006 with annexes.

statement to the Commission that he has seen the letter dated May 21, 1997. He further states that "the diagnoses contain so many histopathological terms unknown to me that I would have discussed them with a pathologist at the department, I know I have never done that." Reith has otherwise referred to a memo written by Sudbø in 2000, in which Sudbø consistently uses the "I" form in his description of the handling of the data material. In light of this, the Commission cannot trust Sudbø's assertions relating to Reith's alleged central role in the handling of data files, see also the letter of February 20, 1996 from Sudbø to the Cancer Registry, rendered above, and the letter from the Cancer Registry to Jon Sudbø of March 22, 1996.

The Commission has considered whether Sudbø and/or Reith may have misunderstood and mixed up the number of samples with the number of patients, etc. The Commission finds this not very likely. The Commission here finds reason to refer to Johannesen's letter of May 21, 1997, which in the Commission's opinion should not give room for misunderstandings and misinterpretations. In any case, the letter should have caused a thorough reevaluation on their part which could have brought any misunderstandings to light.

The Commission has furthermore questioned whether the material from Gade and, as the case may be, the Odontology has subsequently been supplemented, for example by material from other patients having been collected and brought into the project, for example from Gade, the Odontology, private practices or other channels. The Commission has not found any grounds at all for this being the case. The Cancer Registry drew the same conclusion. This is in fact confirmed also in the dissertation and in the articles. Such a supplement is also contrary to expectations for more practical reasons.

Sudbø's allegations that he obtained supplementing information as regards complete personal numbers do not, in the light of Puntervold's, Reith's and the Cancer Registry's statements, appear as probable, and they are also somewhat surprising and contradictory in light of his other allegations that it was Reith who was responsible for the handling of data files between Gade and the Cancer Registry and the arrangement of the data material otherwise. On this background the Commission maintains its clear understanding that the patient number stated is not correct.

Accordingly, the Commission finds that there was probably no access to linked data from more than 144 persons, in the best case from 207 persons.

Exclusion of patients with erythroplakias (red patches in the oral cavity)
In the PhD dissertation is stated that 21 out of 263 patients were excluded initially from analyses of survival because they had erythroplakias. Erythroplakias is far more serious than white patches, and also occurs much more seldom. It should be noted that these persons, i.e. persons with erythroplakias, probably were

included in a subsequent study with 37 erythroplakia patients which formed the basis for a scientific publication.[33]

The Commission finds that the figure 263 cannot be correct. Whether there were 21 persons with erythroplakias in the basic material seems uncertain to the Commission. Johannesen of Gade, however, has stated that erythroplakias were not registered at Gade. If so, this means that patients with erythroplakias must originate from the Odontology. And Sudbø himself stated to the Commission that the erythroplakias originated exclusively from the Odontology. The Commission is in some doubt that 21 of the 61 persons who came from the Odontology had erythroplakias, but this can nevertheless not be excluded due to the missing documentation of these patients. The article that reported the follow-up results from 37 erythroplakia patients does not state from where the patient material (which allegedly was collected in 1988-2000) originates.

However, Sudbø has later on, in comments to the preliminary draft investigation report, maintained that the erythroplakias originate from the Odontology. He has stated that a relatively large material of erythroplakias existed there, because Hanna S. Koppang for many years had taken an interest in these lesions. Sudbø has stated that "the original 21 erythroplakias, originally submitted as leukoplakias, but classified as erythroplakia because they originally had been described as erythroleukoplakia." As mentioned, the Commission is in some doubt about this explanation. In addition, the Commission has reviewed the list of the 63 persons coming from Oslo, without finding any references to diagnoses of erythroplakia.

Even if the Commission does not manage to document that 21 persons did not have red patches in their oral cavity as stated in the dissertation, there is nevertheless such considerable doubt and uncertainty related to this material that it gives reason to concern for whether it is correct at all. This particularly applies in light of other findings in this case, which will be accounted for below.

Classification and reclassification of patients with white patches in their oral cavity – the inclusion and exclusion process
In figure 1 in New England Journal of Medicine 2001 and figure 5 in the dissertation (se figure 1 in the report) is stated that 242 patients with white patches in their oral cavity (leukoplakia), originally were included in the study after the alleged exclusion of 21 persons with red patches in their oral cavity (erythroplakias).

The Commission refers to the account above in which is determined that it is not likely that this figure is correct, i.e. that one has not had access to 242 persons with dysplasia diagnosis. The real figure is

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[33] 33 Sudbø J, Kildal W, Johannessen AC, Koppang HS, Sudbo A, Danielsen HE, Risberg B, Reith A. Gross genomic aberrations in precancers: clinical implications of a long-term follow-up study in oral erythroplakias. J Clin Oncol. 2002 Jan 15;20:456-62.

144 or in the best case 207. The Commission nevertheless finds reason to discuss the actual facts which are alleged by Sudbø et al. in the dissertation and articles.

The key item at this stage of the study was to decide which patients met the criteria (the inclusion criteria) to be made part of the planned ploidy study, i.e. a so-called inclusion process. Before starting a research process, it is usual to state inclusion and exclusion criteria for the research participants, i.e. which patients have the qualities to be studied, and which have qualities that mean that these persons cannot be included in the study. It is important that this inclusion process takes place according to certain criteria determined beforehand and that can be checked, in order to avoid inappropriate selection.

To find which patients with dysplasia diagnosis met the inclusion criteria, the patients were checked against three key criteria (see figure 1):

1. Dysplasia classification: One tissue block from each individual patient had to be classified to see which *type* of dysplasia the individual patient had. The patients were then to be divided according to the criteria mild, medium and serious dysplasia.

2. Prior or simultaneous cancer diagnosis: Patients who had or had had oral cancer were to be excluded. This was because it was change/transformation from white patches to cancer over a certain time interval which was to be studied.

3. Insufficient data material: Persons for whom sufficient data were not available had to be excluded.

1) Dysplasia classification (the reclassification): It appears from the three articles and subsequent articles that the classification was made by *four pathologists* who reclassified tissue samples from *each individual patient*, according to guidelines prepared by the World Health Organization (WHO). New England Journal of Medicine 2001 page 1270 states for example: "All histological sections were subsequently reevaluated by four pathologists according to the guidelines of the World Health Organization." According to how the procedure is described, it must be understood that the reclassification took place blindly (without knowing the patients' identity, diagnoses and the like), and that the pathologists worked independently of one another. This is because the classification was based on assessment, and because it was a key element in this study that the classification became as correct as possible.

It is therefore correct, as is stated in the articles and the dissertation, that patients in relation to whom one disagreed on the diagnosis grading, had to be excluded. In the articles and the dissertation is stated that altogether 46 patients had to be excluded for this reason. In Oncology 2001 the figure is 45 (see figure 1), but this is explained in the dissertation by an "outlier" having been excluded, but the Commission cannot see that anything was mentioned about this in the article. Sudbø states that it was deleted by the editor.

This reclassification process is described in detail in J Pathol 2001, in which one also directly compared the classifications of the individual pathologist.

It is a fact that the four pathologists referred to are

- Gisle Bang, Gade's Institute
- Hanna Strøm Koppang, the Odontology
- Anne Christine Johannesen, Gade's Institute
- Bjørn Risberg, the Radiumhospitalet

This appears explicitly from Oncology 2001 in which these four persons are listed with names in acknowledgements. There is no doubt that all of them are qualified pathologists. It is also a fact that the original dysplasia classifications in no way were so sure that they could be used in the inclusion process. This means that originally all patients had received a dysplasia diagnosis, but Gade and the Odontology were and are very clear that the grading was flawed and not directly applicable for scientific purposes. In other words, there was an obvious need for a blinded reclassification for scientific purposes. This need was at an early stage in fact underlined explicitly to Jon Sudbø by the department manager, Professor Jahn Nesland, who is a pathologist himself. Nesland states to the Commission that such a reclassification for scientific purposes normally is performed blindly with two independent pathologists who afterwards have a so-called consensus meeting to compare results in order to arrive at an agreed dysplasia classification. Thus, it is a fact that it was a question of reclassification of the entire material these four were to make. One may ask why one allegedly used four pathologists instead of the customary two. A possible answer is that the classification becomes more certain the more independent pathologists are used for classification.

However, the Commission cannot see that the information that the reclassification was made by four pathologists is correct. Admittedly, Gisle Bang was central in the collection of the original material. Nevertheless, it is a fact that Gisle Bang did not make a reclassification of all the material. In the best case, he can be deemed to have participated in the original, but for scientific purposes obviously flawed classification of parts of the material from Gade, but not by far the whole material.

The same applies to Hanna Strøm Koppang. Admittedly, Koppang was central at the collection of the original material, but she has hardly participated in the reclassification of the material. In the best case she can be deemed to have contributed to the original, but for scientific purposes obviously flawed classification of the material from the Odontology, but not by far the whole material.

Anne Christine Johannesen has in a sense participated in a reclassification of material from Gade, i.e. 144 of the patients. However, she has not classified the material from "all" the patients. But Anne Christine Johannesen is unable to understand that she is supposed to have participated in a reclassification also of the Gade material. She states to the Commission that it was a fact that her task was to quality-assure the material they had delivered to Sudbø and Reith, such that her responsibility was limited to doing so. She believes this appears clearly from the aforementioned cover letter dated May 21, 1997 to Jon Sudbø with a copy to Reith. In Johannesen's letter reference is made i.a. to a letter from Reith to Myking of May 15, 1996, in which as mentioned reference is made to the fact that the linkage with the Cancer Registry data had identified a transformation rate from dysplasia (white patches in the oral cavity) to cancer of as much as 50%. This sensational finding is also emphasized by Sudbø in a letter to Anne Christine Johannesen of December 9, 1996. However, Johannesen points out that the high transformation rate of 50% which Sudbø and Reith had referred to, is based on several obvious errors, for example that those that had received dysplasia diagnosis had a prior or simultaneous cancer diagnosis, such that no transformation can be determined. It further appears that Johannesen did not make a careful reclassification based on the criteria mild, moderate and severe dysplasia.

In this context it should also be noted that the Cancer Registry this year has found it very difficult to comprehend why Sudbø and Reith in December 1996, several months after the Cancer Registry's linkage, asked Johannesen to revise/reclassify diagnoses relating to patients who should have been excluded based on the information they had received from the Cancer Registry, and based on far more data per patient than Johannesen had any possibility to possess.[34]

Nor did Bjørn Risberg see himself as one of the four pathologists. Risberg states to the Commission that when he read this section in the article, he assumed that this concerned four pathologists which were unknown to him, and that his task had only been to quality-assure the reclassification performed by others. Risberg cannot recall how many samples he classified, i.e. that he may have classified between 150 and 242 samples. The Commission finds it surprising that Risberg has not been informed that he was one of the four pathologists. In the dissertation it is only Risberg who is thanked for having made a reclassification, whereas Koppang is thanked for histological classification.

In his comments to the preliminary draft investigation report, Sudbø writes among other things that he is "entirely incapable of understanding that the original dysplasia classifications could not be used for scientific purposes. This is the first time that I have heard that this point has been raised. However, it was discussed whether one should have had consensus meetings and calibration by pathologists prior to the classification. I objected strongly to this, because the clinics base their treatment on routine diagnostics, not especially constructed diagnostic procedures and assumptions. Everyone involved in this project,

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[34] Letter from the Cancer Registry to the Commission of May 9, 2006.

including Reith, Bryne and A.C. Johannesen, agreed that there was much to say for this view." Furthermore, Sudbø asserts that the term "reclassification" was consistently used only to designate that different pathologists, independent of one another, had classified and graded the dysplasias.

The Commission does not have confidence in Sudbø's explanation on this point, since in the articles and the dissertation he so clearly and unambiguously refers to a "reclassification" by four pathologists in line with the WHO's guidelines. It is also a fact that Johannesen was not "involved in the process" by discussing the planning and the challenges as the impression seems to be by Sudbø's explanation.

The Commission finds that the reclassification was not performed by four pathologists in the manner described in a series of articles and the PhD dissertation.

In the articles and the dissertation it is alleged that 46 patients were excluded because one did not obtain consensus on the grading of the dysplasia diagnosis. There can hardly have been any consensus meeting or the like, when in the best case it is only one pathologist who classified the entire material. None of the four stated pathologists have been participating in any consensus meeting or the like. Sudbø states that there was no consensus meeting between these pathologists, but that there was consensus in the form of "conformity of opinions". The Commission also finds such conformity of opinions to be entirely unlikely, as long as the total material hardly was assessed by four pathologists.

The allegation that 46 patients were excluded due to failing agreement among four pathologists regarding dysplasia grading thus appears as unfounded and erroneous.

The Commission has not found anything to underpin that the figure 46 is correct. The Commission has no alternative figure to put forward, however, as long as the material has not been reclassified in a proper manner for scientific purposes, and as long as the Commission believes that the patient basis was far smaller than what is stated.

2) Preceding or simultaneous cancer diagnosis: The articles and the dissertation state that 36 patients were excluded from the study because they had a simultaneous or preceding cancer diagnosis. The point of the study was exactly to study which patients, after having received a dysplasia diagnosis, *at a later point in time* received a cancer diagnosis. Consequently, persons who had a simultaneous or preceding cancer had to be excluded. The Commission's review of available data files shows, however, that the number of patients with a simultaneous or preceding cancer diagnosis must have been far higher than what is stated in the articles and the dissertation. Reference is again made to Johannesen's letter of May 21, 1997 to Sudbø and Reith in which precisely this point is emphasized, i.e. that a far higher number of patients had a preceding or simultaneous cancer diagnosis, and that this was something Sudbø and Reith obviously had overlooked.

Johannesen's data file annexed to the aforementioned letter shows that at least 47 out of 144 persons should have been excluded for this reason. The Cancer Registry's own investigations also conclude that this figure should be far higher. The Cancer Registry believes that at least 12 out of 63 persons from the Odontology and 76 out of 156 persons from Gade ought to have been excluded for this reason.

Sudbø states that this information is new to him, and he is not quite able to comprehend it. He raises the question of whether all preceding or simultaneous cancer diagnoses are referring to oral cancer or related (tobacco-conditional) cancer. He points out that it is of limited interest whether a patient has had colorectal cancer, cervical cancer, or melanoma prior to oral cancer. Finally, he raises the question of whether an updating of the Cancer Registry's data base has taken place as regards registered cancer incidents since 1995/96.

The Commission here refers to the letter from Johannesen that shows that already in 1996/97 one was aware of all cases of preceding or simultaneous cancer. For the avoidance of doubt, the Commission has in addition compared the Cancer Registry's list from 1996 with the Cancer Registry's data from this year.[35] For the most part there is consistence both as regards cancer dates and localization (the type of cancer diagnosis). There are a few deviations regarding the referring letter date, but nothing of substantial importance. Thereby, it is also clear that this concerns preceding or simultaneous oral cancer, and not other types of cancer. In this context, the Commission has checked the localization code entered for each patient to see if the latter is the case.

Accordingly, the Commission finds that the number of persons with preceding or simultaneous cancer diagnosis was far higher than the number stated in the articles and the dissertation. This is a very serious matter, which clearly and isolated seen entails that the research results cannot be considered as valid.

3) Flawed data material: Finally, in figure 1 is stated that 10 patients were excluded because one did not have appropriate data or material from these patients. Based on the discussions above, this number must be considered as being unlikely low. Reference is here made to the fact that the material from the Odontology (63 patients) was not linked by the Cancer Registry, and therefore should have been excluded for that reason, if not before. Reference is also made to the fact that one only had material from 144 patients from Gade.

It is obvious that far more patients should have been excluded because one did not have sufficient material to include them in the study.

In this context, the Commission finds reason to point out that Johannesen in a letter of May 21, 1997 remarks that "there is no conformity between the original diagnosis and biopsy answer and that the latter must apply". Furthermore, it is remarkable that Jon Sudbø in an email to Johannesen of December 11, 2001 writes that "The dysplastic material is unadulterated in the sense that it does not include patients with

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[35] List: "Sammenlign_kreft96_og_06xls" ["Comparison cancer96 and 06xls"]

simultaneous or preceding carcinoma, neither in their oral cavity or UADT otherwise. The not dysplastic material is restricted, 46 cases."

Based on this, the Commission finds that there are so many important errors and flaws in the inclusion and reclassification process that the resulting outcome is not credible. The Commission is of the opinion that the errors in the reporting are serious.

The inclusion of 150 patients in the ploidy study

According to the articles and the dissertation, altogether 150 patients were included in the study itself. This ploidy study, which is the experiment proper in the PhD project, was made in 1998-99.

At that time Sudbø had used up his four years as a research fellow, and also a last supplementary year. He was then left without any fellowship salary. His closest superior, Professor Håvard Danielsen, PhD, then proposed that Sudbø should use the method developed by Danielsen to analyze the material that Sudbø had in his possession. Sudbø accepted this, and Danielsen arranged for six months of salary funds from the Radiumhospitalet as well as the assistance of a laboratory technician. Sudbø alleges that he himself had taken the initiative to this image analysis already in 1998, but had not got access to the equipment because his oral cavity project was not a prioritized project at the department.

Although it is unlikely, based on the preceding discussions that there were 150 patients that met the inclusion criteria, the Commission has nevertheless found reason to investigate the ploidy analysis in more detail. This is of importance for the clarification of whether the obvious errors that so far have been discovered are due to sloppiness and incompetence or scientific dishonesty.

The Cancer Registry's investigation: In its investigation, the Cancer Registry refers to Johannesen's "limited reclassification" showing that one was left with a maximum of 85 patients only who met the inclusion criteria, divided on mild cysplasia (58), moderate dysplasia (18) and severe dysplasia (9). (In addition, 8 persons were given the diagnosis dysplasia, 4 hyperplasia, 4 preceding cancer, 43 simultaneous cancer – in aggregate 144). Correspondingly, table 1 in New England Journal of Medicine 2001 shows a distribution on mild dysplasia (49), moderate dysplasia (57) and severe dysplasia (44) – in aggregate 150. In other words, the numbers stated in the article do not at all correspond with Johannesen's classification. Nor does the grading stated in the New England Journal of Medicine 2001 correspond to the list Jon Sudbø according to the Cancer Registry received from the Cancer Registry in 1996, and which had the following division: dysplasia 4, mild 38, moderate 22 and severe 99, in aggregate 163.

The Cancer Registry believes that this number of patients that could be included could have been 79 as a maximum, particularly because of preceding or simultaneous cancer diagnosis (77 out of 156). That is to say close to half of what is stated in the articles and the dissertation.

In spite of the unambiguous findings of the Cancer Registry, based on its own investigations and Johannesen's independent classification, the Commission has nevertheless found reason to make some investigations of its own. This is connected with the fact that the Cancer Registry's conclusion that no doubt manipulation and fabrication of data was involved, was quite sensational and serious.

The Commission's own investigations of patient lists and the ploidy analyses: The Commission has been given access to several lists which apparently contain data from 150 patients who probably were included and studied in the ploidy analysis which took place in 1999. It is a fact that the lists comprise 150 observations, i.e. registrations. It is also probable that someone (see in more detail about this below) has analyzed at least 150 blocks/samples/preparations/monolayers. Based on the aforementioned investigations and findings, the Commission raised two entirely central and specific questions:

1.   Was the ploidy analysis performed on 150 different persons, or may it be that several analyses originate from the same person?

2.   Do the persons included and studied really meet the inclusion criteria?

With the help of available data lists and comparisons between them, and comparisons with among other things data from the Cancer Registry, it has been possible to obtain precise documentation of which patients were actually included, including these patients' disease history (i.e. whether they met the inclusion criteria).

The lists and the patients form the basis for the three mentioned articles, the dissertation and several subsequent publications, i.a. New England Journal of Medicine 2004, and have therefore been in the very center of attention for the Commission, see annex 3.

The Commission will here discuss these lists in more detail. In particular three lists are of interest:

- **L-29.** This list is assumed to be the original list used in the study, and which the Commission has received from Danielsen. As head of section, Danielsen obtained it from the archives in 2006 on the Commission's request. It is noted on the list that it was produced in April 1998. The list is assumed to form the basis for the PhD project, including New England Journal of Medicine 2001.

- **Rawdata.** The Commission has received this list from Reith. Reith has stated that he had not seen this list until 2006, when he asked Ruth Puntervold for it in connection with this case. Puntervold is supposed to have received the list from Sudbø in 2005 in connection with Bjørn Risberg's wish to measure the preparations again, see 5.3. It is in harmony with, and is probably based on, L-29. According to Reith, the data list forms the basis for New England Journal of Medicine 2004, which again in all essentials is based on New England Journal of Medicine 2001.

- **Sudbø8.** The Commission has received this list from J. Jack Lee at MD Anderson, who again received it from Sudbø. Lee is the bio statistician who ran the analyses which form the basis for New England Journal of Medicine 2004. According to Lee, this list is the basis for New England Journal of Medicine 2004.

These three lists are in harmony with each other, which means that there is a preponderance of probability that they originate from the same patient material. However, the individual lists contain more or other registrations. The Commission has not found any basis for these lists not forming the basis for the analyses which again form the basis for the publications in i.a. New England Journal of Medicine 2001 and 2004. Nor has Sudbø or others submitted any patient lists that deviate essentially from these lists.

The rawdata list comprises preparation/block/sample numbers which make it possible to obtain information from the Cancer Registry. This list is, apart from block numbers, identical to Sudbø8.

The table in annex 4 shows the 150 observations (records) which the Commission has assumed formed the basis for the article in New England Journal of Medicine 2004. The first column is a continuous numbering of observations as listed in Rawdata and Sudbø8. The second column comprises unique persons. Each observation in Sudbø8 comprises one or several preparation numbers (block numbers). These preparation numbers were linked to the same person apart from two members in observation 51, which proved to belong to two different persons. This observation is therefore listed twice. The third column is the preparation number itself. This is blanked out for reason of personal data protection. There were 8 observations on the rawdata file for which there were no preparation number. For these 8 "missing" is noted in the column. Then follows a column showing the year when the preparation (the sample/biopsy) was taken. The three next columns are from the file which the Cancer Registry delivered to Sudbø in 1996. Then follow three columns from Sudbø8. The column "year leukoplakia" should be corresponding to the column "year preparation". Finally there is a column showing whether preparations for ploidy classification had been made.

The Commission has had access to dates for leukoplakia diagnoses and dates for cancer. These coincide entirely with Rawdata and Sudbø8. Moreover, it should be mentioned that information on age and tobacco is entirely in conformity on the two files. This documents that these two files must originate from the same patient basis.

This comparison also documents which persons did not meet the inclusion criteria, since the date of oral cancer is before the sample (block/preparation number) was taken. The Commission shows that 69 of 150 observations should have been excluded for this reason. The comparison further documents that there are only 64 different persons on the list which we could document as not being contrary to the inclusion criterion. It should be noted that the year has been removed from the block number in the rawdata list. An indication of

the year could have contributed to someone having discovered the latter error. On the other hand, the year is stated on L29, without anyone having discovered discrepancies with the inclusion criterion.

The comparison shows that none of the dates in Sudbø8 agrees with the data from the Cancer Registry. This means that the dates in Sudbø8 are fictitious. The Commission's comparison of the Cancer Registry's list from 1996 and the Cancer Registry's list from 2006 are in all essentials concurrent, so that an error at the Cancer Registry is excluded. And the Cancer Registry has also made a thorough investigation of i.a. all relevant referring letters, etc.

Totally there is reference to ploidy preparations for 69 out of 150 observations (65 different persons) in the rawdata list. Observation number 51 in the rawdata (patnid_re=51) is listed twice since this observation in rawdata had two preparation numbers which proved to be two persons.

The Commission's comparison shows the following:

- The rawdata list and Sudbø8 comprise 150 observations made up of a maximum of 140 persons
- Of those 150 observations only maximum 81 observations meet the inclusion criterion
- Of the 150 observations there are only 69 observations in which there is a reference to a ploidy preparation
- Of the 150 observations there are 23 observations in which the year of death is prior to the year of leukoplakia (the year in dateopl). This means that the patient was dead before the diagnosis allegedly was made.
- No observations with block numbers originate from the Odontology in Oslo.

The list "All original blocks and HE biopsy specimens linked to ploidprep and L29 series" moreover shows a connection between block numbers and list numbers (L31 etc) for the observations in which there is a ploidy preparation (probably ploidy classification). In all there are 167 observations. The Commission has a list with a variable/column, "place", which shows whether the block is from Gade or from the Odontology.[36] This is defined based on block number or list number. As regards the list L47 there is verification with block numbers. The Commission's list shows the following:

	Observations	Unique block numbers
Missing	4	4
Gade	97	80
The Odontology	66	42
In total	167	126

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[36] List: "Alle_ploidi_060606.xls"["All ploidy 060606.xls"]

Consequently, there are 167 ploidy preparations, of which 126 only are unique block numbers. This means that there are unique and thus valid ploidy preparations for a maximum of 126 persons. This can be compared with that, according to Sudbø himself, 196 persons were processed statistically where ploidy classification existed in J Pathol 2001. In other words, this statement hardly agrees with the actual facts.

It is worth noting that only 69 out of 167 ploidy numbers can be linked to L29/Rawdata/Subø8. This means that a ploidy analysis has only been made on 69 (and not 150) of the block numbers existing in the rawdata list, which is the basis for the New England Journal of Medicine 2004.

For this reason, the Commission has seen no point in making a new ploidy analysis of the raw data, since the raw data is so obviously flawed.

Sudbø has reacted to this, and has among other things referred to one of the persons who classified remembering to have received about 150 blocks. Danielsen also believes to have seen a tray with approximately 150 blocks. The Commission would remark to this that one has probably classified approximately 150 blocks, such that those who classified, i.a. Wanja Kildal, probably believed that it concerned the number of persons stated in the articles. But the fact is that it involved many duplicates and many persons who should have been excluded, i.a. due to preceding and simultaneous cancer diagnosis.

The Commission has calculated an age distribution from the file Sudbø8 (which form the basis for New England Journal of Medicine 2004) and compared this with the age distribution for the original data from Gade and the Odontology. For Gade two schedules have been made: 1) Based on a list produced by A.C. Johannesen, which via block numbers is linked with Cancer Registry data, and 2) Based on the file that the Cancer Registry delivered to Sudbø in 1996. Table 2 shows the result for three age groups. Age for Gade and the Odontology is age when the biopsy was taken. Age in Sudbø8 is not defined in more detail on the file itself, but in an email to J. Jack Lee of MD Anderson (who made the analyses) Sudbø writes that this is age at "time at initial diagnosis". Thus we can assume that there are the same age definitions in the files when age for diagnosis is stipulated as age when the biopsy was taken. Moreover, it is difficult to see which other age it could be in Sudbø8. The table shows that there are far more persons in the age group 65-78 in Sudbø8 than in the other files, also when they are joined. Thus there is a distinct discrepancy between the file which forms the basis for New England Journal of Medicine 2004 and the files which form the basis for this.

Table 2: Age distribution in data file for New England Journal of Medicine 2004 (Sudbø8) compared with the age distribution in data file delivered from the Cancer Registry in 1996 (the Oslo Odontology [62 persons] and Gade [163 persons] and lists from Gade [142 persons].

Basis	Canc.Reg.06	Canc.Reg.96	(Canc.Reg.06/ Gade list 98)			JJL06
	Oslo	Gade1	Gade2	Oslo+Gade1	Oslo+Gade2	Sudbø8
Age*						
30-64	40	79	68	108	119	32
65-78	13	59	53	66	72	112
79-95	4	25	19	23	29	6
Total	57	163	140	197	220	150
Lacking	5		2	7	6	0
Total	62	163	142	204	226	150
Average age	56.8	63.5	63.3	61.4	61.8	68.9
New England Journal of Medicine 2001						68.9
New England Journal of Medicine 2004						68.9
*Age for Oslo and Gade: Referring letter year minus year of birth; Age for Sudbø8:age in file						

The Commission has also had access to the first file which was sent to the USA for New England Journal of Medicine 2004 and compared this with the last file. Table 3 renders the results for some observations.

Table 3. The last 28 records from the first and last file which formed the basis for the article in NEJM 2004.
Both files contain 150 records (lines) numbered continuously at patnid from 1 to 150.

Updated rawdata (first file)					Sudbø8 (last file)				
patntid	age	tobacco	yr leukoplakia	yr cancer	patnid	age	tobacco	yr leukoplakia	yr cancer
123	80	3	1993	2000	123	80	3	1993	2000
124	74	3	1993	2001	124	74	3	1993	2001
125	74	3	1993	1994	125	74	3	1990	1991
126	65	2	1994	2000	126	65	2	1994	2000
127	76	3	1991	1993	127	76	3	1989	1990
128	55	3	1985	1989	128	55	3	1985	1986
129	66	2	1982		129	66	2	1982	-------
130	72	3	1994	1995	130	72	3	1991	1992
131	75	4	1989	2000	131	75	4	1989	2000
132	76	3	1985	1986	132	76	3	1983	1984
133	65	1	1990	1993	133	65	1	1987	1988
134	73	3	1997	1998	134	73	3	1994	1995
135	66	4	1987	1988	135	66	4	1984	1985
136	51	3	1995	1997	136	51	3	1990	1994
137	69	3	1989	1990	137	69	3	1986	1987
138	69	1	1991	1992	138	69	1	1983	1988
139	78	3	1994	1996	139	78	3	1991	1993
140	67	3	1997	1998	140	67	3	1994	1995
141	78	2	1989	1989	141	78	2	1986	1988
142	63	3	1995	1997	142	63	3	1993	1994
143	78	3	1991	1995	143	78	3	1988	1990
144	64	2	1994	2002	144	64	2	1994	2002
145	63	3	1993	1995	145	63	3	1988	1991
146	74	4	1993	2002	146	74	4	1993	1995
146	67	3	1996	1998	147	67	3	1994	1997
148	74	2	1985	1989	148	74	2	1983	1985
149	65	1	1990	1992	149	65	1	1987	1988
150	81	2	1994	2000	150	81	2	1994	1994

Conspicuously many dates have been changed from the first to the last file. Even more conspicuous is that the year for the leukoplakia diagnosis has been changed, whereas the age for the leukoplakia diagnosis is unchanged.

Accordingly, the Commission finds that the lists to which the Commission has had access and which form the basis for the PhD project, are not correct. Neither dates, number of patients, nor other checkable observations agree to any reasonable degree with the published data and results. The Commission has tried different approaches and has made a series of other comparisons of lists, sample numbers and patient identities, etc., but the conclusion has always been the same. Neither dates for the leukoplakia diagnosis nor

the date for cancer agree with the corresponding dates in the Cancer Registry, and it is difficult to find any other explanation than that dates and lists to a large extent have been fabricated. This finding is in harmony with the Cancer Registry's independent internal investigation. This finding is also in harmony with the Commission's pointing out of flaws in the patient basis.

The ploidy analysis

After having decided which patients should be included in the study (allegedly 150) and which had to be excluded (allegedly 113) via the dysplasia classification, the next step was to carry out the experiment itself. This consisted of classifying the samples/blocks/monolayers from patients included in the study, to see which degree of dysplasia (mild/moderate/severe) and which type of lesion the patient had by the help of a genetic analysis – a so-called ploidy analysis; graded according to diploid/tetraploid/aneuploid. The point was to determine whether a relatively simple DNA analysis of the white patches could predict the likelihood of subsequent development of cancer.

At this time a ploidy classification was made at the Radiumhospitalet, i.e. a measurement of the amount of DNA (hereditary material) by an image-analytic machine. The analysis machine then makes a DNA histogram which draws up a person classification. The classification is subjective, even if the purpose of the machine analysis is to make it as reliable as possible and by that objective.

New England Journal of Medicine 2001 states on page 1272 that "all specimens were coded, and DNA histograms were classified in a blinded manner by four observers." In J Pathol 2001 there are three. This is in spite of the routine at the hospital being that the classification was to be made blindly by only two independent persons.

To the Commission it has been somewhat unclear who these four independent persons were. Jon Sudbø explains that it was Wanja Kildal, Håvard Danielsen, Jon Sudbø himself, and partly Albrecth Reith. Bjørn Risberg did not take part in this classification, although he was obviously qualified for it. Risberg himself has been somewhat surprised that he was not included in this classification. Reith explains that he understood that Wanja Kildal and Håvard Danielsen made the classification. Reith further states that he himself some time later made an analysis of the histograms to see if he "was in line with WK and HED's analyses", but obviously he does not see himself as one of four observers, in that he refers to the fact the dissertation does not state anything about "four" observers.

It must be assumed that Wanja Kildal, who had been trained by Håvard Danielsen, was qualified to classify the samples, in the same way as Håvard Danielsen. Wanja Kildal states that she classified all the samples she received from Jon Sudbø. After Wanja Kildal had classified the samples, she showed Håvard Danielsen the first 30 classifications, in order that he could check if she had done it correctly. This

was done by Danielsen. Sudbø firmly believes that Danielsen also classified the remainder of the samples. He also alleges that a consensus meeting took place between Danielsen, Kildal and himself. Danielsen on his part is certain that he did not classify the other 120 samples, and that no consensus meeting ever took place. Based on Reith's own statement, the Commission finds that Reith can hardly be considered as one of the four alleged observers. Whether and to which extent Jon Sudbø classified the material, seems rather unclear to the Commission. If Sudbø did classify, it is doubtful whether the classification was blinded inasmuch as Jon Sudbø and Albrecth Reith probably had access to and knew the patients' identity and diagnosis. Principally, Sudbø denies this about the non-existing blinding. Alternatively, he alleges that since both he himself and Reith "had had access to and good knowledge of the contents of the background file from the Cancer Registry … the non-existing blinding should in such case apply also for him [Reith]." However, Reith denies that he had such access to the data material, and has accounted for this in a way the Commission finds credible. On this point, the Commission will comment that in most observation studies a "blinded" classification may be and is performed even if those who carry out the classification could have cheated by opening the blinding. In normal circumstances, one has sufficient trust in the researchers who carry out the study.

Accordingly, the Commission finds that the ploidy analysis hardly took place as described in New England Journal of Medicine 2001. On the other hand, this is not an item of crucial importance to the validity of the results, since the Commission is convinced that the samples which were analyzed comprised several duplicates, and that several blocks originated from patients who should have been excluded from the study. The ploidy analysis itself then appears to the Commission as pseudo valid, since the results were not linked with the correct number of patients that could be included.

The research result

The results of the ploidy analysis compared with cancer development, i.e. the research result itself, was astounding. The question was to which extent the ploidy in cells from white patches could be used as a sign (a predicative indicator) of future oral cancer, which is a very serious form of cancer.

Sudbø et al could show that patients with aneuploid lesions had a particularly poor prognosis, by approximately 90% developing oral cancer during a five years' follow-up. At the same time patients with diploid lesions had a very good prognosis, by only 5% subsequently developing cancer. For patients with tetraploid lesions the probability of transition to cancer was a little above 50%. Thereby Sudbø had confirmed his hypothesis and arrived at a very good method to predict oral cancer for persons with white patches.

Based on the above, this sensational research result can no longer have credibility.

4.2.8   Other errors and flaws

In the following, the Commission will summarily point at certain other errors and defects which together and alone entail that the credibility of the three stated articles and PhD dissertation is considerably reduced.

Defective blinding

A weakness in this study is that the researcher in charge (i.e. Jon Sudbø) had full access to all patient data. This is a weakness because the study according to the articles and dissertation was to be a blinded historic prospective study, i.e. a study in which by going back in time one may follow patients' future development. Blinded means that those who made the ploidy analyses and dysplasia classification were not to know whether the patient subsequently got cancer or not. In this case whether patients who had first got the diagnosis white patches in the oral cavity (dysplasia) at a later point in time developed cancer. Such a transformation and its frequency would be able to say something about the extent to which the ploidy in cells from white patches could be used as a sign (a predicative indicator) of future oral cancer. But because the study was an historic prospective one, one in fact had the answer on one's hands, by knowing how many patients developed cancer. Therefore, the person who was in charge of the research itself, Jon Sudbø, should not have had access to the patient information before the classification of dysplasia and ploidy had been completed, what both he and Reith according to Sudbø himself had.

As previously mentioned, the Commission will at this point state that in most observation studies a "blinded" classification can be and is performed, even if those who perform the classification could have cheated by opening the blinding. Under normal circumstances, one has sufficient trust in the researchers who carry out the study so that the requirements as to blinding are not as stringent as in randomized studies, for example.

However, the Commission will point out that a lack of blinding as regards Sudbø, may contribute to explaining how he was able to manipulate for example the ploidy analysis in order to show positive research results, for example by including patients who had had oral cancer (and who for that reason should have been excluded). It should be noted here that another, alternative, explanation is that the analysis results have been manipulated later on.

Misleading reporting

New England Journal of Medicine 2001 page 1271 reads:

There was no fixed follow-up program for persons with a diagnosis of dysplasia and who did not have cancer at this time. Admittedly, some patients were probably summoned to a check, but this was not done systematically, and it was not necessarily a question of an annual follow-up within the framework of a "program" either, as is the clear impression the article gives. It is also a fact that such a follow-up did not comprise all patients. However, Sudbø maintains that the program did not form part of his scientific project, but that at this time the routine at both Haukeland and Clinic for Oral Surgery and Oral Medicine was to set up a follow-up agreement a year later if a biopsy had been performed. Since this routine was institutionalized, Sudbø believes it is justified to call it a "program".

However, the Commission finds this argumentation doubtful. The Commission furthermore is very much in doubt that no patients dropped out of the follow-up program.

The Cancer Registry has also difficulties in understanding that which is described in the article, and clearly expresses that this cannot be correct, as this is not the way things function in reality. On the other hand, obviously no others, for example coauthors, reacted to this description.

On this basis, the Commission finds that the way this has been described in the article in the best case is misleading.

Errors as regards smoking and alcohol habits

Table 1 and the text in New England Journal of Medicine 2001 refer to smoking and alcohol habits and a follow-up program for dysplasia patients. New England Journal of Medicine 2001 states on page 1272:

"Patients with confirmed use of tobacco or alcohol were given standard oral and written information on risk factors for oral cancer, and this information was repeated at each follow-up visit. Data on tobacco use were reconstructed from the medical records or by the use of telephone interviews, in which the patients were

asked about their use of tobacco at the time of the initial diagnosis of oral leukoplakia (no history of tobacco use, former use of tobacco, or use of tobacco at the time of the initial diagnosis."

The Commission has discussed this information with specialists with knowledge of this. It should be noted here that the Cancer Registry has had access to *and has reviewed* all the referring letters.

The material from Gade comes from hospitals all over Western Norway, from Møre og Romsdal in the North to Rogaland in the South. There is no information that the clinics who sent biopsies to Gade's Institute participated in any form of scientific study which concerned smoking and alcohol habits or the like. Detailed information on smoking habits is therefore seldom stated on the pathologists' referring letters, a fact confirmed by the Cancer Registry's review.

As did these experts, the Commission believes that the information that all patients who smoked or used alcohol were advised, either orally or in writing, of the hazards they exposed themselves to, is erroneous. It may happen that clinicians – to a larger or smaller degree – informed their patients of such facts. Sudbø or other persons involved in the research project have clearly enough not been providing such type of advice, and it is impossible that they can have had access to this information, and they also never had any personal contact with the patients.

In New England of Medicine 2001 it is alleged on page 1274 that the information about smoking habits was achieved from the journals of 100 patients. A further 37 patients were telephoned and gave such information to Sudbø et al. There is nothing to indicate that such information can have been retrieved from the patient journals as alleged. There are no grounds to believe that Jon Sudbø, for example, has had access to the medical journals of these patients. Copies of the journal would in such a case have had to be sent to Sudbø, or he may have traveled across Western Norway and retrieved information that way. In any case, medical journals will often lack systematic information on smoking (not a smoker, present smoker, previous smoker). Correspondingly, there is little reason to believe that the information on alcohol use has been accessible in any systematic way.

Sudbø states that he himself, together with Puntervold and Reith, called around to the 37 patients for whom smoking data allegedly were missing, to supplement the data basis. However, no log for the carrying out of such telephone interviews exists. Sudbø has explained that information obtained in such telephone conversations was noted down on loose bits of paper and/or plotted directly into the data file. Puntervold and Reith, however, state that they have not called any patients about this. Moreover, the Commission finds it improbable that one called to a sufficient number of patients about whom there must have been a need for supplementary information. It should also be noted that 48 persons were dead in 1995, that is before the time

information allegedly was retrieved (see table in Annex 4). In other words, the Commission does not trust Sudbø's explanation on this point.

In this context, it must be remarked that Sudbø as the main supervisor contributed with data on smoking to one of his research fellows in 2004/2005. However, the fellow has this year, right after the submission of the PhD assertion, based on this case reevaluated the data. The fellow found that there is no conformity between the data on smoking the fellow received, and the data on smoking stated in New England Journal of Medicine 2001, although the raw material is the same. The fellow found, i.a., that *all* the patients in the material received from Sudbø are smoking, whereas in New England Journal of Medicine 2001 there are patients (27/150) who have not had any consumption of tobacco. The fellow's dissertation has for this reason, among others, been retained, and will probably be retracted.

The data used in New England Journal of Medicine table 1 is incompatible with data one may reasonably expect that there was access to at that time. The Commission is in strong doubt that Sudbø has had access to complete sets of data about smoking habits like he has given the impression of in i.a. New England Journal of Medicine 2001 and to his research fellow. The information on smoking and alcohol habits appears to the Commission to be partly fabricated.

In this context, the Commission finds reason to note that, in spite of flawed data, New England Journal of Medicine 2001 page 1277 states that the information on smoking was taken into account in the multivariate analysis, but that it did not influence the results. At the same time, it is stated that reliable data for alcohol use were not available for more than half the patients, and that the use of alcohol was therefore not included in the analysis. Both these points have been accounted for in an elegant and convincing manner, and reinforce the reader's belief in this researcher, partly because the researcher appears as honest and thorough, and partly because he points out possible weak parts of his own study. This elegance in the presentation of research results may be a possible explanation of why no one found any particular reason to question and critically check the publications, more than what was done at some times.

Double publication?

The Commission has put the question of whether the two articles in New England Journal of Medicine 2001 and J Pathol 2001 have so many similar features that they must be considered as a double publication of the same research results in contravention of good research practice. The main results in New England Journal of Medicine 2001 and J Pathol 2001 are coinciding, but nevertheless such that the J Pathol article provides more details as regards the four pathologists' dysplasia classification.

The Commission has submitted the question of a double publication to Reith and Sudbø. Reith acknowledges that it is a defect that there is no cross reference. However, he refers to the article in J Pathol 2001 to a much larger degree than New England Journal of Medicine 2001 discussing in-depth methodological and conceptual matters, of particular interest to pathologists. Thus Reith believes that the article has its own value and that it was not possible to include these matters in the article which was to be published in New England Journal of Medicine. Sudbø refers to the subject double publication being a relevant problem at the time of publication, which was discussed, but they concluded that it was within what was acceptable. Reith has also referred to the lack of cross reference having its explanation in both manuscripts being submitted simultaneously, and that New England Journal of Medicine does not allow cross references to articles which are submitted only. A cross reference was according to Reith regrettably forgotten at the later time when the manuscripts following several rounds finally were accepted and ready for publication.

The Commission is in doubt regarding this point, but has concluded that there was no obvious double publication. The Commission nevertheless chose to comment on this point to show that the question has been considered.

Confusion or manipulation of pictures?

Media has paid lots of attention to the fact that the same picture in New England Journal of Medicine 2001 appears twice, but is stated to represent two different patients. This is the basis for the expression of concern that the editors of the journal have published. It is a fact that it is the same picture, but in different sizes. This is an obvious error. Jon Sudbø has admitted this, but alleges that it was due to an excusable confusion.

The Commission has not considered this to be an important point and for that reason not pursued the matter further.

4.2.9 Summary

To make a thorough and checkable investigation of the actual facts has been a very difficult task to perform. This is partly because the research is advanced and based on a large amount of data which can only be understood with knowledge of technicalities linked to specialized patient studies.

Another reason that this type of investigation is very difficult is the lack of precise documentation of all steps in the research process. This problem is important and not unusual, and not unique to this case. Furthermore, sets of data and lists will exist in several versions with different names and it may be difficult to know which changes have been done, by whom and when. The investigation is further made difficult by the fact that for reasons of personal data protection and secrecy one cannot use person-unique identification of the

information, but must make use of sample numbers, preparation numbers or block numbers, and many data files are entirely without identification. In this section, the Commission will try to summarize section 4.2.7. As stated, the Commission has concentrated its investigation on the article in New England Journal of Medicine 2001, because it is definitely the most important publication in Jon Sudbø's research career. It forms the basis for several contemporary and subsequent original articles, i.a. a similar article published in the same journal in 2004 (New England Journal of Medicine 2004).

The main analysis and the main findings both in the 2001 and 2004 articles in New England Journal of Medicine are based on the same patient material consisting of 150 patients. Samples from these 150 patients have i.a. been classified according to DNA ploidy in samples from dysplasias in the oral cavity. This ploidy classification was in the 2001 article shown to be a strong predictor of future cancer development. This finding was reinforced in the 2004 article with a further follow-up and 11 new cancer cases.

The Commission has received the data file (Sudbø8) from MD Anderson, with confirmation that it forms the basis for the New England Journal of Medicine 2004 article by reproducing Figure 3B among other things.

This file agrees with a list the Commission has received from Reith. This list again agrees with a list (L29) which the Commission has received from Head of section Danielsen and which is produced in 1998.

Having made a thorough evaluation based on the Commission's own review of a large number of lists and data files (Annex 3: Files_lists.doc), and the Cancer Registry's extensive retrieval of all referring letters with relevant dysplasia diagnoses, and the reconstruction of Sudbø's data material with the linkage that the Cancer Registry made in 1996 with a file of 226 persons (63 of 226 patients who came from Oslo were not connected at the time because they lacked personal identification numbers) as the starting point, the Commission has found the following fundamental problems with the central patient material of 150 patients used in i.a. New England Journal of Medicine 2001 and 2004 articles, and in a series of other articles:

The same patient appears several times

The 150 patients do not exist, in the sense that as far as the Commission can see, it is a matter of a maximum of 141 patients. The reason for this is that some patients are represented by several samples which in the aggregate give the number 150. Letting a patient reappear with several dysplasia samples is contrary to the description in the articles, and does not give any scientific meaning in this context. These replicas of persons are therefore invalid, and should have been excluded from the file. The Commission has not been able to determine the precise number of replicas (this is because 8 of the 150 lack block numbers on the Reith list),

but has concluded that the number of different persons in the file is at the most 141.

Failing exclusion due to simultaneous or previous cancer

Among the patients who the Commission has been able to identify in the Sudbø8 file there is a large number of patients who should have been excluded because they had had oral cancer prior to or simultaneously with the dysplasia in question. The Commission has found that at least 69 persons cannot be included, as they already had oral cancer at the time of the referring letter.

Ploidy analyses have not been made for all patients

The Commission has only been able to retrieve ploidy analyses for 69 of the observations (65 different persons) in the Sudbø8 file. This is based on lists of ploidy analyses which Puntervold has obtained from the Sudbø material (Gade/the Odontology). A far greater number of ploidy analyses (>150) have been performed, but the same patients appear several times.

Age distribution is not correct

The age distribution in the original material from Gade and the Odontology does not correspond to the age distribution in the material which formed the basis for the New England Journal of Medicine 2004.

Out of these four fundamental problems, it is number 2 which appears as the most serious, and which the Commission with a high degree of certainty can determine, because it is underpinned by independent information from several sources. When more than half of the central patient material is excluded, also all the results in both of the New England Journal of Medicine articles fall to pieces as well as all further research based on these.

4.2.10 Main conclusion

The Commission finds that Jon Sudbø has not had access to the number of patients which he states to have had, including that the dysplasia classification and inclusion process have not been made in an honest way such as described. The Commission in particular refers to the fact that out of the 150 of allegedly 263 patients included in the study, more than half of the included patients should have been excluded due to a preceding or simultaneous diagnosis of oral cancer.

It is thus evident that both articles in New England Journal of Medicine and all further research based on the same material cannot be correct or be based on reality, apart from being based on qualified guesswork. Publications based on this raw material must for that reason be retracted, see furthermore section 4.4.

There can be two explanations for the errors discovered:

- An unfortunate combination of excusable errors and misunderstandings, as well as failing competence, alertness and thoroughness.
- Scientific dishonesty, i.e. the fabrication and manipulation of research data and consciously misleading research reporting.

The Commission finds that the errors and defects discovered are too many, too large and too obvious to be ascribed to excusable errors, incompetence or the like. The Commission finds that data have been manipulated and fabricated, and probably adapted to the findings one wanted to arrive at.

The Commission finds that Jon Sudbø has been alone in the manipulation and fabrication of data. The Commission will revert to this in more detail, however, including other players' role in the continuation, see in particular Chapter 5.

Based on the account given above, the Commission finds that there is scientific dishonesty on the part of Jon Sudbø related to the PhD dissertation, New England Journal of Medicine 2001, Oncology 2001 and J Pathol 2001.

4.3 After the presentation of the thesis

After having defended his thesis at the University of Oslo on March 9, 2001, Jon Sudbø continued his scientific activity, within the same field, i.e. oral cancer. This resulted in a series of scientific original articles, reviews, readers' letters and the like, which were published currently in several renowned medical journals.

In connection with the publication of the research results in New England Journal of Medicine 2001, Reith and Sudbø in a letter to the Norwegian Board of Health wrote that the research results should lead to a changed practice as regards screening and treatment of patients with white patches in their oral cavities in Norway. By this, many patients could be saved, was the allegation. They also mentioned the need for a prospective study. The Board of Health replied that they did not have any opportunity to reorganize treatment practice in Norway, due to a lack of resources, among other things. The Board of Health also referred to the fact that support for a prospective study would had to be applied for to other quarters.[37]

Concurrently with the publication of New England Journal of Medicine 2001, Dagens Medisin *[an independent newspaper for the health sector]* on April 26, 2001 reported a "Breakthrough in the battle

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[37] Letter from Reith to the Acting Health Director of January 11, 2001. Letter from the Norwegian Board of Health of April 17, 2001. Letter of April 23, 2001 from Reith and Sudbø to the Norwegian Board of Health.

against oral cancer". "Between eighty and ninety percent of all cancer developments can be predicted by chromosome analysis" Sudbø is supposed to have stated to the newspaper.

In an editorial written by Scott M. Lippmann and Waun Ki Hong (with whom Sudbø subsequently initiated a collaboration) in New England Journal of Medicine 2001 in the same edition of the journal, the study was called an important progress as regards the assessment of the risk of oral cancer for patients with leukoplakia. The editorial states:

> "The new molecular data have important implications for the standard of care of patients with oral leukoplakia. Local management ranges from watchful waiting to resection with widely varying margin widths, depending on histologic and clinical features. Molecular information can redefine the assessment of the risk of oral cancer and even guide treatment, with the one important caveat that the molecular results involving patients with severe dysplasia in the studies we have discussed may have been confounded by the small numbers of such patients and the likelihood that they underwent more rigorous surgical procedures than did the patients with mild or moderate dysplasia. It is time to establish standard molecular assays to help plan the management of oral leukoplakia. … Confirmation of the completeness of resection, close monitoring, and chemoprevention trials would be appropriate approaches for patients deemed at high risk on the basis of molecular assays, including patients with hyperplasia.[38]

In 2001, Jon Sudbø applied to the Cancer Society for money for the project "Early diagnosis and treatment (chemo prevention) of early stages of oral cancer" for 3 years. The application was denied.

At the same time he also applied to Health and Rehabilitation *[a foundation granting money to voluntary organizations and efforts to improve physical and mental health in Norway]* via the Cancer Society for funds to the project "Protocol – prevention of oral cancer". He obtained funds for this project for the years 2002-2004. He was later on also granted funds for 2005.

In 2001-2002, Jon Sudbø also came in contact with American researchers within the same specialist field. Reith and Sudbø met with Scott Lippmann from MD Anderson personally for the first time in November 2002 during a conference in Frankfurt. According to Reith, it was Lippmann who suggested the collaboration which was initiated. This collaboration led to several publications in leading medical journals, as for example New England Journal of Medicine in 2004 and The Lancet in 2005, see section 5.3.

Thus, in 2001 Jon Sudbø had started the work with what was to become part of a big project application to the National Cancer Institute (NCI) in the USA. The project was granted 10 million dollars, of which most went to MD Anderson in the USA.

The Commission will revert in more detail to this PROTOCOL study in section 5.3, but will first account for the consequence of the main conclusion in 4.2.10 for other publications.

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[38] Lippman S. M., Hong W. K. Molecular Markers of the Risk of Oral Cancer. N Engl J Med 2001; 344:1323-1326.

4.4 Other publications

In the following, the Commission will deal with the articles suffering from such substantial defects and doubts that they cannot be considered valid, and for that reason should be retracted. The Commission has reviewed the 38 publications which resulted from an individual search for Jon Sudbø in January 2006 in the PubMed data base,[39] see Annex 1. The Commission realizes that this publication list is not exhaustive, but has nevertheless found reason to base itself on the list as it in all probability comprises the most important works. Sudbø has not commented on the draft for this section in particular, but refers to the comments cited above.

The Commission refers to the main conclusion in section 4.2.10 and to the general account of retraction of scientific publications in section 3.6.

In the following, the Commission will deal with the publications in which the Commission has found errors and the like. Of most interest are Sudbø's original articles, totaling 12. By an original article is meant that the article comprises original research results which are not presented previously. It is such articles that are the most important and most meritorious within the research communities. When reviewing the PhD dissertation which contains 6 original articles, the Commission previously found that three original articles must be retracted, whereas no errors were found in three original articles, cf section 4.2. In this section will be held that a further 5 original articles must be retracted or at least be subject to an expression of concern for their validity. This means that 8 out of 12 works appear as more or less invalid. Out of Sudbø's original articles there are only the three first articles in the PhD dissertation that are found not to contain errors, see section 4.2.5, as well as a less sensational article published in Oral Disease 2003.[40] Accordingly, there is a basis for stating that the essential parts of Jon Sudbø's scientific production suffers from errors and flaws caused by scientific dishonesty.

The publications which are not found to contain errors, apart from four original articles, mainly concern lesser reviews and letters of less scientific value, as well as works in which Jon Sudbø has only been a coauthor, i.e. publications which mainly have been prepared by others.

- Sudbø J, Warloe T, Aamdal S, Reith A, Bryne M. Diagnostikk og behandling av forstadier til munnhulekreft [Diagnosis and treatment of oral precancerous lesions] Tidsskr Nor Laegeforen 2001;121:3066-71. Overview article.

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[39] www.pubmed.gov
[40] Sudbø J, Reith A, Florenes VA, Nesland JM, Ristimaki A, Bryne M. COX-2 -expression in striated muscle under physiological conditions. Oral Dis 2003:313-6.

The article is an overview article that summarizes results from Sudbø's articles included in his PhD degree. The article is therefore based on raw data which the Commission has found are manipulated and partly fabricated, cf section 4.2. The article must therefore be retracted.

- Sudbø J, Kildal W, Johannessen AC, Koppang HS, Sudbo A, Danielsen HE, Risberg B, Reith A. Gross genomic aberrations in precancers: clinical implications of a long-term follow-up study in oral erythroplakias. J Clin Oncol. 2002;20:456-62. Original article.

  The article asserts that it is based on analyses of a material that has not been used in previous publications. The PhD dissertation states that material from 263 persons was collected and that 21 of them were excluded from the original study due to having red patches in their oral cavities (erythroplakias). It is persons with erythroplakia that have been studied in this study. In the method part it appears that the material comprises 57 samples with human biological material from 37 patients with erythroplakia collected in the period 1988-2000.
  The Commission here refers to the fact that it has not found any indications that these raw data exist.
  In its other findings, the Commission has found that so much doubt is linked to whether these raw data in fact exist that it is reasonable to apply a large question mark to this article.

- Sudbø J. [DNA ploidy analysis--a possibility for early identification of patient with risk of oral cancer] Läkartidningen. 2001;98:4980-4. Review.

  The article is a review of Sudbø's own research results. That means that it is based on the raw material which the Commission has based itself on, cf section 4.2. The article must for this reason be retracted.

- Reith A, Sudbø J. Impact of genomic instability in risk assessment and chemoprevention of oral premalignancies. Int J Cancer. 2002;101:205-9. Review.

  The article is a review which is based on Jon Sudbø's earlier research results, which again are based on raw data that are manipulated and partly fabricated, cf section 4.2. The article must for this reason be retracted.

- Sudbø J, Reith A. Which putatively pre-malignant oral lesions become oral cancers? Clinical relevance of early targeting of high-risk individuals.
  J Oral Pathol Med. 2003;32:63-70. Review.

  > The article is a review which is based on Jon Sudbø's earlier research results, which again are based on raw data that are manipulated and partly fabricated, cf section 4.2. The article must for this reason be retracted.

- Sudbø J, Reith A. When is an oral leukoplakia premalignant?
  Oral Oncol. 2002;38:813-4; author reply 811-2. Debate contribution.

  > The article is a review which is based on Jon Sudbø's earlier research results, which again are based on raw data that are manipulated and partly fabricated, cf section 4.2. The article must for this reason be retracted.

- Sudbø J, Ristimaki A, Sondresen JE, Kildal W, Boysen M, Koppang HS, Reith A, Risberg B, Nesland JM, Bryne M. Cyclooxygenase-2 (COX-2) expression in high-risk premalignant oral lesions. Oral Oncol. 2003;39:497-505. Original article.

  > The study is based on an analysis of the raw data which form the basis for parts of the PhD work and which are manipulated and partly fabricated, cf section 4.2.
  > The article must therefore be retracted.

- Sudbø J, Bryne M, Mao L, Lotan R, Reith A, Kildal W, Davidson B, Soland TM, Lippman SM. Molecular based treatment of oral cancer.
  Oral Oncol. 2003;39:749-58. Review.

  > The article is a review based on the raw data collected during the PhD degree work and which are manipulated and partly fabricated, cf section 4.2.
  > The article must therefore be retracted.

- Sudbø J, Lippman SM, Lee JJ, Mao L, Kildal W, Sudbo A, Sagen S, Bryne M, El-Naggar A, Risberg B, Evensen JF, Reith A. The influence of resection and aneuploidy on mortality in oral leukoplakia. N Engl J Med. 2004;350:1405-13. Original article.

The article is based partly on the raw data collected in connection with the PhD degree work and which the Commission has found are manipulated and partly fabricated, cf section 4.2. See in particular under section 4.2.7 in which the Commission accounts for the comparison of the data list used in New England Journal of Medicine 2001 and New England Journal of Medicine 2004. In addition the article is based on a follow-up of the same patients which did not take place. The latter fact is partly admitted by Sudbø to the Commission.
The article must therefore be retracted.

- Sudbø J, Reith A. The evolution of predictive oncology and molecular-based therapy for oral cancer prevention. Int J Cancer. 2005;115:339-45. Review.

  The article is a review of earlier research and based on material used both in New England Journal of Medicine 2001 and New England Journal of Medicine 2004. The Commission has determined that this material is manipulated and partly fabricated, cf section 4.2. The article must therefore be retracted.

- Sudbø J, Samuelsson R, Risberg B, Heistein S, Nyhus C, Samuelsson M, Puntervold R, Sigstad E, Davidson B, Reith A, Berner A. Risk markers of oral cancer in clinically normal mucosa as an aid in smoking cessation counseling. J Clin Oncol. 2005;23:1927-33. Original article.

  The article is based on allegedly newly collected material from 275 persons. This material is supposed to have been collected via dental clinics. The Commission has tried to obtain documentation showing that these raw data in fact exist. The Commission has been in contact with, i.a., the dentists that are listed as coauthors because they had assisted in collecting material from their patients. These dentists confirm that they were asked by Jon Sudbø to collect scrapings from patients. However, they only collected 10-20 samples each. It therefore appears as rather unlikely to the Commission that Sudbø can have had a complete data material from 275 patients. The Commission also finds it quite unlikely that these patients were enlisted in a program for smoking cessation with a further follow-up. No indications have been found that such a program was implemented. The cited dentist colleagues have difficulties in understanding that such a program existed. The Commission's

assessment must also be seen in light of other deviations from good scientific practice which the Commission has detected.

Jon Sudbø has admitted that cotinine level was not measured for all patients that participated in the study. The latter fact alone means that the study cannot any longer be considered valid. The journal has published an expression of concern relating to this article and the editors state that with the exception of Sudbø and Reith, none of the coauthors participated in the preparation of the manuscript and they therefore do not meet the authorship criteria. In the Commission's view, the article should be retracted.

- Sudbø J. Novel management of oral cancer: a paradigm of predictive oncology. Clin Med Res. 2004;2:233-42. Review.

   The article is a review of earlier research in which reference is made to the original raw data collected in connection with the PhD dissertation and which are manipulated and partly fabricated, cf section 4.2. The article must for this reason be retracted.

- Sudbø J, Lee JJ, Lippman SM, Mork J, Sagen S, Flatner N, Ristimaki A, Sudbo A, Mao L, Zhou X, Kildal W, Evensen JF, Reith A, Dannenberg AJ. Non-steroidal anti-inflammatory drugs and the risk of oral cancer: a nested case-control study. The Lancet. 2005;366:1359-66. Original article.

   The article is based in its entirety on fabricated raw data and is for that reason already retracted. These facts have been admitted by Sudbø. The Commission has for this reason not spent much time on investigating this article. However, the Commission got access to the correspondence between Jon Sudbø and J. Jack Lee of MD Anderson. Thereby the Commission detected how these new raw data came about, see section 5.3.

5. Possible explanations

5.1 Introduction

In Chapter 4 the Commission found that the raw material which formed the basis for the main part of the PhD project, is manipulated and fabricated. This contributes to a series of later publications having to be disregarded. The Commission has also found several cases of data manipulation and fabrication in the subsequent scientific career of Jon Sudbø. The Commission found that this was due to scientific dishonesty on the part of Jon Sudbø.

The comments that Sudbø has given to the draft report did not provide the Commission with reason to make substantial changes to the preliminary conclusions reached during the investigation.

The Commission has not found grounds to believe that others have participated in manipulating and fabricating research data or in any other way committed scientific dishonesty, as this is defined in the recently adopted Research Ethics Act section 5 (2) (not yet in force).

In its terms of reference, the Commission was asked to seek an explanation of the facts discovered. The Commission has asked itself how these – in retrospect – obvious and gross acts could take place, in collaboration with a series of well qualified coauthors and collaboration partners, and at a renowned research institution.

In introduction, the Commission will emphasize the obvious, namely that there will always be a possibility for the dishonest person to cheat and defraud others. No system is water tight in this respect, and this also applies to Norwegian research. The question here is first and foremost whether it is possible to identify factors that have contributed to the acts discovered by the investigation.

The Commission has by no means any foundation for drawing definite conclusions about what caused these circumstances. However, the Commission finds reason to point out certain factors which may contribute to illuminate how and why "things turned out as they did". To the Commission, a part of the explanation is that a series of "unfortunate" factors occurred simultaneously, and these must be seen in context to illuminate the case.

Criticizable facts are summarized in Chapter 7.

5.2 The PhD project and further research

The Commission has got the clear impression that Jon Sudbø acted relatively freely and independently, both as a research recruit (research fellow) and researcher. This impression is confirmed by several of Sudbø's

colleagues. Reith explains this by Jon Sudbø appearing as an exceptionally clever PhD candidate and that he was also relatively experienced and not as young as many of the other research fellows. In his comments to the draft report, Sudbø objected to Reith's description of Sudbø's relatively free role, and in that connection pointed out that he had little or no experience as a researcher at the time when he started his PhD project. Sudbø conceives this as an attempt by Reith to disclaim responsibility.

The Commission's total impression based on the information that has come to light during the investigation, is all the same that Reith seems to have had an active but nevertheless relatively superficial role as a supervisor. Reith seems to have observed what went on and discussed this underway with Sudbø, without having been involved in the analyses and treatment of data as such. Nor can he have carried out random checks or suchlike to ensure quality. It seems as if Reith has replied to questions from Sudbø, but not himself put control questions or the like. However, Reith has provided contact with both the Cancer Registry and Gade and thus been a key initiator and door opener. Then he has allowed Sudbø to work independently with the data analyses themselves. The Commission here refers to the fact that Sudbø as mentioned got access to both the material and the patient data. This made it possible to manipulate and change around data lists, blocks and analysis results without anyone discovering it. In light of the description of the facts in Chapter 4, the Commission finds reason to state that Reith should have been more alert as a supervisor and followed Sudbø's treatment of the data material more closely and not least checked to a larger extent certain factors that are erroneously described in publications in which he is listed as a senior author.

Another element which may provide a certain explanation of the facts that have been discovered during the investigation, is that no formal approval or review of the project exists at all. No permissions, evaluations from the Norwegian Board of Health (now: The Directorate for Health and Social Affairs), the Data Inspectorate/the Norwegian Social Science Services or the Regional Committee for Medical Research Ethics exist, something which also contributes to elude further investigations and quality assurance of the project. As mentioned above, this flaw is something for which Sudbø himself, Reith and the Radiumhospitalet must assume the responsibility.

However, the Commission will underline that there is no reason to believe that such failing compliance with the formalities has been a conscious strategy in order to be able to carry on a dishonest research activity. The explanation of these circumstances seems mainly to be that sufficiently effective and good enough routines have not been in place as regards the control and supervision of research projects at the Radiumhospitalet. The fact that Sudbø did not have a formal employment relationship to the Radiumhospitalet, is evidently enough no acceptable explanation. The Commission has indeed found (section 4.2.3) that the research took place under the auspices of the Radiumhospitalet and that this institution had the overall responsibility for Sudbø's research activities. Even if instructions and control bodies existed at the

institution, it does not seem as if these were satisfactorily implemented. The project was not submitted to the Protocol Committee, for example, and no one saw reason to question it. Researchers associated with the department indeed seem to have had a relatively relaxed relationship to the formalities. This applies in relation to the retrieval, delivery and treatment of human biological material and sensitive patient information, recommendations from the Regional Committee for Medical Research Ethics, licenses for data processing and dispensation from the duty of secrecy. The Commission has also found reason to express doubt as to whether the organization of this research project has been defensible. This organization is an institutional responsibility.

Furthermore, it may seem that when someone was worried that quality routines may not have been followed, this was not followed up by the management or colleagues, maybe because one did not have any system or tradition regarding this type of warning. It must be noted here that one person retained all the documentation from the contact with Sudbø, exactly because this person had his/her suspicions. This was at the end of the 1990's. However, the person concerned did not want to get into a difficult "whistleblower position" and therefore refrained from making further investigations.

Concerning the relationship to coauthors, what happened in connection with the publication of New England Journal of Medicine 2001 is apparently characteristic of Sudbø's choice of and collaboration with coauthors in subsequent works. The Commission here relies on written and oral feedback from almost 60 coauthors. A possible explanation of why the research cheating was not discovered earlier may partly be found in the fact that the coauthors mainly appeared as sub-suppliers or as senior guarantors (i.e. senior researchers who primarily played a central role on the overall level (idea, planning, compilation and the like) and who by reason of their professional weight have contributed to giving the works a professional legitimacy). Moreover, the authors have mainly related to Sudbø. In other words, there has apparently not been much communication "horizontally" between the various coauthors or research groups. Most of the communication took place via Sudbø. Sudbø has pointed out that he did not prevent communication between the coauthors in any way, but the Commission cannot see that he has contributed to or has called for such a contact between the coauthors. In this way, Sudbø has been in control of which set of data, which information and the like the individual coauthor possessed. It is worth pointing out that this information probably was limited. None of the coauthors seem to have had access to the underlying data material and patient data. They have probably not seen this as a natural or obvious part of the assignment given them either. However, such a distribution of work is not an unusual phenomenon in medical publications – a fact that obviously must be taken into account in the assessment of whether there is a basis for criticizing the coauthors. As an

example of this, reference is made to the collaboration between Jon Sudbø and J. Jack Lee in connection with the article which was published in New England Journal of Medicine 2004:

> At one time Sudbø sends a radically changed file to J. Jack lee at MD Anderson[41]. The file shows "data cleaning along the way" as expressed by Lee in an email to Gunnar Sæter of January 13, 2006 after this case had become known. The Commission has compared two files: a) "sudbo8" which is the file that was used in the article in New England Journal of Medicine 2004 and b) "updated rawdata" which according to Lee is the first file he received from Sudbø for the 2004 article. The comparison was based on the variable patnid (patient number) which is on both files. The Commission has included the following variables: age, tobacco, year leukoplakia and year cancer. An excerpt of the 150 observations is provided in Table 3 in the report. As previously mentioned, it is conspicuous that "year leukoplakia" has changed from the first to the last file without age having changed. This is mathematics which does not tally if age is age at leukoplakia. In an email from Sudbø to Lee of August 8, 2003 Sudbø writes: "One point: the age of the subjects have been recalculated to what they were at the time of initial diagnosis". The latter ought to mean that age at leukoplakia is what is concerned, see section 4.2.7.

On a general basis, the Commission finds reason to note that many research fellows and researchers will have the opportunity to manipulate and even fabricate raw material, without either supervisors or coauthors being able to discover it. Research is indeed to a large degree based on trust, as it necessarily has to be. It is important and necessary that researchers have trust in one another, that one may trust that what others supply is genuine. The employer must be able to trust its employees, and journals must be able to trust researchers. Research participants and the public in general must also have trust in researchers. But such trust cannot be without limits. There must be a certain control and sound skepticism, because at one time or another warning lamps should start to flash thereby causing checks and controls to be made. But the fact that very few researchers find it obvious that any of their partners will fabricate research data may explain something of that which in retrospect easily may appear as failing alertness.

On the other hand, there are, as the Commission sees it, certain descriptions in the articles which more people should have reacted to. This may be coauthors, supervisors, superiors, critics, colleagues and others. The Commission would in particular refer to the account of the reclassification of the dysplasias which allegedly was made by four independent pathologists,[42] as well as ploidy analyses which allegedly also was made by four independent observers.[43] This was neither usual nor the case. The Commission also refers to the allegations relating to the reporting to the Cancer Registry, a follow-up program for patients and the allegation that one was in possession of almost complete smoking data. These are matters which one maybe

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[41] Dataliste: "updated_rawdata_renamed_sudbo8.xls"
[42] "All histologic sections were subsequently reevaluated by four pathologists according to the guidelines of the World Health Organization";NEJM 2001 page 1272.
[43] "All specimens were coded, and DNA histograms were classified in a blinded manner by four observers"; New England Journal of Medicine 2001

in retrospect should be able to say that someone should have discovered, or at least put a question mark at – both inside and outside the professional community at the institution, taking into account that this information was published in the internationally most renowned medical journal, namely the New England Journal of Medicine, which is remarkable for Norwegian researchers. At the same time, it must be admitted in the coauthors' defense that the supervisor as well as coauthors in conversations with the Commission has had difficulties in and used much time to reconcile themselves with articles, which they themselves had taken part in and felt ownership to, being in fact based on fabricated data. The latter shows how unbelievable and unusual fabrication of research data in Norwegian research communities is perceived to be by researchers.

5.3 In particular about the NCI application and Lancet article

During the period 2000-2001 Jon Sudbø and Albrecht Reith as mentioned got the idea for a new and larger study. Also this study was related to the prevention of oral cancer, but this time it was to be a prospective study in which they were to follow patients forward in time. The study was named PROTOCOL, which stands for *Prospective randomized trial on preventing oral carcinomas from oral leukoplakia.*

In 2000/2001 Jon Sudbø prepared a protocol. Following internal discussions at the Radiumhospitalet it was suggested that the protocol should be translated into English, as the potential was considered to be big, and that it ought to be an international study. Jon Sudbø translated the protocol himself in the summer 2001.

At the same time Sudbø and Reith applied for support to the project from Health and Rehabilitation via the Cancer Society. They were granted funds for the years 2002 until December 31, 2005.

In 2002-2003, Jon Sudbø and Albrecht Reith were fairly often in the USA for seminars and the like. In that connection they came into contact with Scott Lippmann and the professional community relating to MD Anderson, as well as Andrew Dannenberg of Weill Medical College at Cornell University. This contact gradually developed into a professional collaboration. This collaboration resulted in agreement to apply to National Cancer Institute (NCI) for funds for a large study. Sudbø's PROTOCOL study was to be included as an element in this study. The application was submitted to NCI in the summer of 2003.

Jon Sudbø has stated that at the same time, that is in the beginning of 2003, he started working on the data list which later on was to form the basis for the article in The Lancet, which has a certain connection to the NCI study by a preliminary version of these data being included in the application according to Lippmann, but without having a central role. Jon Sudbø has stated to the Commission that the basis for this data list is a series of data published under the auspices of the Cancer Registry and the Norwegian Institute of Public Health, among other institutions. These are data relating to tobacco habits, cancer and other relevant factors.

Thus, he made a table of cancer incidents in various age groups and various periods of time, and then entered tentative dates of birth for fictitious patients. In other words, the list is based on qualified guesswork. The list was supplemented by fictitious use of NSAID, various types, various doses, various time intervals, such that one could see how various factors were distributed on patients and the control group, with different risk of getting oral cancer based on smoking habits. Sudbø has stated that this originally was meant as a pure simulation data base – i.e. fictitious number experiments. Nothing has come to light to indicate that other persons knew about this.

The further time and sequence of events are somewhat unclear to the Commission. However, the Commission has had access to the comprehensive correspondence that took place between Jon Sudbø and the bio statistician at the MD Anderson, J. Jack Lee. This correspondence documents in detail how Jon Sudbø managed to produce the fabricated data file which forms the basis for the Lancet article. The communication between Sudbø and Lee per email with data files attached, shows how Jon Sudbø first sent a fictitious data file to J. Jack Lee.

The Commission will not discuss this in detail, but would emphasize that the common theme in the correspondence is that J. Jack Lee points out errors, defects and inconsistent factors in the file he had received. Then Jon Sudbø accounts for how he will have these regrettable errors corrected, errors which according to Sudbø must be based on misunderstandings and other unfortunate circumstances. According to Sudbø's accounts per email to Lee, the correction of errors was made in cooperation with qualified specialists and institutions. An email of January 29, 2005 states, for example:

> "I checked also with the health survey people, who scrambled on Saturday. The confirmed alcohol was not a selection criterion for the original search."

Jon Sudbø writes in an e-mail of March 29, 2005 to Jack Lee:

> "Albrecht and I will be in meetings with CONOR (Cohort of Norway), the consortium which administers the databases of the health surveys. We need to make sure we have documented all there is to document regarding these surveys and how they are linked to other population based disease registries."

Another example of Sudbø demonstrating his will to work day and night with this project and his access to not only competent specialists but also to the registers in Norway is in an email dated June 6, 2005, in which he writes to Scott Lippman, J. Jack Lee and Andrew Dannenberg:

> "Please find attached clean copies of the NSAID paper, cover letter and responses. Jon Mork and I spent most of the weekend at the Cancer registry, checking the number of cases with cancers in different locations of the oral cavity. We have also had a meeting to go over the final drafts of the paper, responses and cover letter."

An e-mail of September 30, 2005 states:

> "Tonight, I have gone through and discussed the commentators viewpoints and the responses from Jack, with epidemiologists at the Cancer Registry. They ([N.N.], lead epidemiologist on Jon Morks 2001 NEJM paper, and [N.N.] also epidemiologist at the Cancer Registry and on the Mork 2001 paper) found the responses to the point, and well placed. In other circumstances, this should get us on dry land with respect to acceptance."

It must be noted that the Commission is of the opinion that it is not likely that these meetings took place, or that Sudbø or any of the mentioned persons had such access to the Cancer Registry and such information.

The application to NCI was granted in March 2004, and the total grant was for approximately NOK 70 million. However, the project is mainly an American project directed by Scott Lippmann and MD Anderson. Jon Sudbø's PROTOCOL project only got a small part of this grant. According to Sudbø, at least NOK 16.5 million was to be transferred to the Radiumhospitalet in the course of a five years' period. He refers to the Norwegian project being one of four projects included in the total application (a so-called Program Project Grant).

In the summer and autumn of 2004, an application was made to the Regional Committee for Medical Research Ethics South Norway (REC-South) and the Data Inspectorate for approval of the PROTOCOL study. The study was approved on August 13, 2005 and October 19, 2004, respectively.

The PROTOCOL study was opened for inclusion of patients in December 2005. When it became known through media that the Lancet article probably was based on fabricated research data, this study was stopped for an indefinite period of time. No patients had then been randomized (included) in the study, but five patients had been through introductory interviews. One patient had according to Sudbø been subject to a surgical biopsy for assessment with a view to randomizing in the study.

The Commission has reviewed the NCI application and the material delivered by Jon Sudbø. The application is based on Sudbø's raw material, which partly consists of fabricated data. There is nothing to indicate that any of the collaboration partners knew of or had any suspicion about this. Jon Sudbø also gives the impression that he can supply data and analyses, which, at least in retrospect, it is relatively obvious that he would not have been able to supply. He also states that he has received public permissions which are obviously fictitious. In this connection, the Commission refers in particular to page 130 of the application, where a series of essential points appear as pure fiction [the Commission's running commentaries are included in square brackets]:

Project 1 leader Dr. Sudbo and Core C Co-Leader Dr. Reith built over a 10-year period the infrastructure that will support clinical Project 1 and its translational interactions with PO1 colleagues at M.D. Anderson and Weill Medical College of Cornell University (Dr. Dannenberg).

- 1993-4: Access to biopsy specimens from all Norwegian pathological departments approved by the Norwegian Cancer Registry (Kreftregisteret), Norwegian Data Protection Agency (Datatilsynet), Norwegian Department of Health and Social Security (Sosial- og helsedepartementet). [This appears in all essentials as pure fiction, cf section 4.2.]
- May 1995: Access to Norwegian Cancer-Registry data for evaluating follow-up of 150 patients with oral white patches. [The date should have been from the beginning of 1996, and the material referred to is partly manipulated and fabricated, cf section 4.2.7].
- ...
- January 1997: Access to this information granted by the Regional Ethical Committee (Regional Etisk Komité), Norwegian Data Protection Agency (Datatilsynet), and the Internal Advisory Board at the Norwegian Radium Hospital (NRH). [All this is incorrect, cf 4.2.4]
- January 1998: Permission from the Norwegian Data Protection Agency (Datatilsynet) to do telephone interviews to get additional epidemiological information regarding smoking and alcohol habits and comorbidity from persons in the study. [Incorrect, cf 4.2.4]
- ...
- February 2003: Epidemiological data on NSAID effects obtained from The Norwegian Cancer Registry and National Health Survey Project. [This is pure fiction.]

In this context should be noted that in the application, Jon Sudbø demonstrates that he has a full overview of the formal procedures which apply to this type of medical research projects.

While the raw data gradually took form through the cooperation with an obviously unsuspecting J. Jack Lee, Jon Sudbø started work on the article which subsequently was published in The Lancet. A draft article was first sent to New England Journal of Medicine, which rejected it twice. The Commission has not been given access to the referee opinions. The article was then finally published in The Lancet in October 2005, after having been through a so-called fast-track referee system. It is worth noting that one of the professional colleagues, who reviewed the article, was highly negative to its publication.

The main results that were subsequently published in the Lancet article[44] are to be found in a power point presentation by Dr. Ernest Hawk of National Cancer Institute in the USA from a FDA hearing in the middle of February 2005.

On April 7, 2005, American health authorities (FDA) warned against cardiovascular side effects of non-selective NSAIDs. European health authorities were more reserved, and the Commission does not know of any changes to the guidelines having been made. According to the press, the Norwegian Medicines'

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[44] www.fda.gov

Agency is giving priority to the case, but as far as the Commission knows no changes have been made to the Norwegian guidelines.

On April 7, Aftenposten *[a major Norwegian daily newspaper]* spent half its first page to report that Ibux (ibuprofen) could lead to heart disease. On May 3, Jon Sudbø is supposed to have stated to Adresseavisen[45] *[another Norwegian daily newspaper]* that he "had to triple check the data because he could not believe they were correct. But they are water tight." Jon Sudbø is unable to understand this press coverage.

5.4 Questions are raised in relation to the ploidy classifications

In the summer 2005, Jon Sudbø was contacted by Bjørn Risberg who wanted to use the raw material on which Jon Sudbø's PhD work was based in another study. In this connection, Risberg repeated parts of the ploidy classifications. It appeared that these in no way agreed with the classifications on which Sudbø had based his work. Risberg, together with Eva Sigstad, pointed this out to Sudbø in a letter dated August 3, 2005, at the same time as they asked to be delivered the data material used as basis. In the letter is stated:

> "We have now made up the results from our automatic measurement of your previous M41 study (on oral mucosa biopsies) and compared with the results you arrived at by automatic measuring. The results are as follows:

	Manual	Automatic	Same ploidy result
Diploid	29	36	18
Tetraploid	15	14	6
Aneuploid	13	7	1
Unsuited/no block no.	24	24	
Total	81	81	25

> There is a large discrepancy between the results. The cause of this can be several:
> 1. wrong rendering of the data in the data bases
> 2. measuring errors; degenerated ("old") material in the automatic measurement
> 3. differences of method between manual and automatic
>
> It is essential to find the cause of the discrepancy. Further automatic measurements will otherwise be without value. We would appreciate hearing your views on this problem."

The Commission will comment to this that Risberg's and Sigdal's measurements are in harmony with the Commission's previous findings under section 4.2.7, in the sense that Sudbø's measurements do not agree when they are checked. In the Commission's opinion, Risberg's and Sigdal's letter is striking, by showing

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[45] http://www.adressa.no/nyheter/sortrondelag/article499549.ece

conformity in only 25 out of 81 measurements. It is obvious that this was a very serious and dramatic accusation against a research colleague in spite of the neutral wording of the letter. In the Commission's conversation with Risberg it appeared that by going further into the raw material for ploidy analyses, it appeared that there was an acceptable conformity between the automatic and manual measurements (which was the original purpose of Risberg's investigation) and that any discrepancy would have to be explained by an exchange of patient identification. According to Risberg, the latter was never clarified.

According to Risberg, Sudbø tried to explain away all of this, and said he would put things straight, but this never happened. Risberg brought this up with the department manager, Jahn Nesland, but nothing came out of it. At this time, the Radiumhospitalet was busy with a very demanding merger with the Rikshospitalet.

Jon Sudbø contests this presentation, and asserts that it was a joint evaluation by himself, Risberg and Reith which led to the reclassification. The Commission does not believe Sudbø's explanation on this point. Also Reith has confirmed that, when Risberg wanted to reclassify the material, it proved difficult to obtain the material from Sudbø. After several reminders, Reith suggested that Risberg wrote a letter to Sudbø to make him give priority to obtaining the material. Finally, Reith did obtain agreement from Sudbø for a meeting, a meeting that Sudbø nevertheless did not attend. The letter from Risberg and Sigstad of August 3, 2005, was unknown to Reith until the case was discovered in 2006.

As regards the choice and involvement of coauthors to works produced in a later phase, where Sudbø was more established as an independent researcher, it is, like in the first phase, typical that these coauthors have had marginal knowledge of the raw material. At the same time, Sudbø surrounded himself with a sufficient number of persons for his project to appear as legitimate. It seems to the Commission as if the coauthors to a large extent were used to legitimize what Sudbø did. It is also typical that the coauthors have had little or no contact among themselves; it is Jon Sudbø who had the full control of what each of them knew, and to what each of them had access. In several cases, the coauthors did not see the final draft of articles, and many of the coauthors were very little involved in the writing of the manuscript itself.

5.5   External factors

In compliance with its terms of reference, the Commission has examined and evaluated whether external causes can have contributed to the breaches of good scientific practice that have been discovered. It may be a question of several causes, as for example the relationship to external cooperating research institutions, including the pharmaceutical industry.

The Commission has evaluated such factors, but has not found grounds for believing that external factors of this nature contributed to the breach of good scientific practice. Nor has the Commission discovered actual facts which have provided reasons to implement more extensive and in-

depth investigations on this point. For example, the Commission does not find it likely that Jon Sudbø produced research data and results commissioned by the pharmaceutical industry.

However, the Commission cannot entirely disregard as a possible cause that Jon Sudbø may have been driven by a wish to satisfy express or unspoken needs, wishes or such like from international cooperation partners, including the pharmaceutical industry, with ensuing honor and recognition, as well as financial support to new research projects. The Commission cannot exclude that interests of this nature to some extent may be an explanation (motivating factor) on the background of the NSAID findings, i.e. the cardiovascular results in the Lancet article. These were results that were sensational and potentially useful to several international main players within the development and manufacture of medicines.

Moreover, there is reason to point out that the type of dishonesty that manifests itself in, i.a., the NCI application and the Lancet article is possible precisely because one cooperated with external institutions with insufficient knowledge of the situation in Norway.

5.6   Flaws in sets of regulations and similar formal types of control

The serious breaches of good scientific practice discovered in this case, are in all essentials breaches of fundamental and unambiguous rules which have existed for a long time. The prohibition against improper manipulation and fabrication of research data is embedded in rules that all researchers must be assumed to be well acquainted with. Therefore, the Commission cannot see that the facts discovered are due to a lack of rules.

On the other hand, the Commission has discovered a series of minor breaches, which in aggregate have contributed to a system in which the breaches of good scientific practice have been allowed to increase without being discovered earlier. But also here it seems, in the Commission's opinion, not to be the lack of rules which is the problem, but rather the individual researcher's and institution's knowledge and practicing of the rules which actually exist. The Commission would here as an example refer to the fact that the PhD project should have been submitted to the Regional Committee for Medical Research Ethics, the Data Inspectorate and the Norwegian Board of Health (now: The Directorate for Health and Social Affairs) and that these are factors no one has reacted to, neither coauthors, supervisor(s) or the management of the institution, nor those who were responsible for approving the PhD degree.

The Commission's findings on this point are in harmony with three studies related to the regulation of medical research:

In its concluding report from 2001, the then National Committee for the Evaluation of Dishonesty in Health Research stated that it was important to have clear rules for good scientific practice. One therefore prepared a guide to the implementation of projects in medical and health research. The committee was concerned that such thematic should form part of the mandatory part of the research education, but believed

that it was just as important that an environment for lifelong learning within research ethics and good research practice should be developed. The committee underlined that the responsibility must lie with education institutions and research environments themselves, but that a central initiative to initiate the process was needed.

This is moreover in harmony with the Nylenna Committee's report from 2005 on the regulation of medical and health research.[46] The Nylenna Committee found that it was not primarily the lack of rules that was the problem, although certain deficiencies existed. The main problem was in the committee's opinion that the rules were fragmented, complex and inaccessible, and that few people had an overview of the set of rules. Simplification and clarification of the set of rules and bureaucracy, as well as making the research institutions more clearly accountable, was the Nylenna Committee's main recommendation for measures.

This approach was also given weight in the preparatory works to the Research Ethics Act.

Thus, the Commission's opinion is that the facts discovered are not primarily related to a lack of rules, but rather that there has been a lack of measures to prevent breaches of good scientific practice through the implementation of simple and effective routines. The latter is first and foremost an institutional responsibility. The Commission has in fact noticed that over the last years there has been an increasing awareness of these factors at several research institutions, among others at the Rikshospitalet/Radiumhospitalet MC, which has implemented a series of measures to guide researchers.[47]

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[46] NOU *[Norwegian Official Reports]* 2005:1: <u>God forskning – better health</u> *[Good Research – Better Health].* Act relating to medical and health research that involves humans, human biological material and health data (Health Research Act)

[47] See <u>www.klinforsk.no</u>. For Aker University Hospital, see <u>www.forskningsjus.no</u>, and for Ullevål University Hospital refer to http://www.ulleval.no/modules/module_123/news_template_avdeling.asp?iCategoryId=664

6.   Possible consequences

6.1   Reflections on consequences for research

The fabrication of research data is counted among the most serious forms of dishonesty in research. When such things occur, it contributes to a general weakening of the society's trust in research. For medical and health research, this doubt about trust may also have consequences for the trust in the health services in more general terms. Such doubt may create insecurity and concern in the population. The researcher community is highly dependant on society's trust, both to ensure that there is a political will to use public budgets to fund research, and that the population will be willing to participate in various research projects.

The Nordic countries have traditionally had a positive reputation within clinical as well as epidemiological research. Based on these countries' health and disease registers, and the possibility of linking data to comprehensive population registers with unambiguous identification opportunities, the Nordic countries traditionally have been sought after as collaboration partners in research projects. For small countries, this type of research collaboration is often of crucial importance for being able to participate in the front of international research. The discovery of dishonesty linked to the use of such registers may weaken the global research communities' interest in collaborating with Nordic researchers.

Research is by nature truth seeking and critical, and has an important task in questioning "established" truths. It is also incumbent on the researcher community to ensure that research activities take place within ethical and honest norms at all times. When dishonest conduct is discovered, the increased focus on norms and the practice of professional ethics provides an opportunity for a renewed discussion of the following up at all levels of research. This case becomes an "eye-opener" which may contribute to an enhanced concentration on the prevention of dishonesty. Even if rules and guidelines for ethical and honest research conduct exist, there is a need for continuous focus on these questions, not least in the researcher education.

The Commission has difficulties in having clear conceptions of whether this case specifically will harm the reputation of Rikshospitalet/Radiumhospitalet MC and the University of Oslo, but the names of these institutions, in particular the first mentioned, will inevitably be linked to Jon Sudbø. It will probably be necessary to allocate resources to show increased openness relating to the practicing of guidelines and quality assurance routines in order to restore and maintain trust. An effort to explain away what has happened will easily be contra productive as regards regaining the trust of the population and authorities. Most people understand and accept that errors and failures can occur now and then.

6.2 Possible harmful consequences regarding the treatment of patients, etc.

The results from the studies have probably been used by many researchers in their works. When the findings referred to are based on incorrect and misleading data, this has in the best of cases caused much wasted work and resources.

Many of the publications deal with the use of diagnostic methods to determine oral cancer and to discuss prognoses for treatment in relation to the time of diagnosis. This may have consequences for the follow-up and treatment of patients. The Commission has not understood it to be its task to discover specific harmful effects. The Commission is aware that this will be a subject for the Board of Health's own investigation.

However, the Commission is aware that the results have been used in discussions on the value of medicines, also as documentation in discussions about retraction of medicines. Also here, the results of Sudbø's research may have had negative consequences, both for the treatment of patients and for the use and sales of medicines. Moreover, the Commission has registered that there are reports in the media that Sudbø's research results have influenced the diagnosis and treatment of certain persons with white patches in their oral cavities in Germany, the UK and the USA. It is also reported in Norwegian media that individual patients have omitted to use painkilling tablets, and rather chosen to live with pain, because it appears from the Lancet article that the tablets entailed an increased risk of cardiovascular diseases, etc.

Evidently, these are very serious matters, and this obvious danger of misleading patients, health staff and researchers with ensuing disadvantages and harmful effects, must have been evident to Jon Sudbø.

7. Criticizable circumstances

7.1 Introductory remarks

In line with its terms of reference, the Commission in this chapter will make a summary of what it found to be criticizable circumstances. For a detailed description of facts and the Commission's evaluation of Jon Sudbø's research, see Chapters 4 to 6. The criticizable circumstances the Commission has discovered are related partly to physical persons and partly to institutions.

The Commission's investigation entailed that 60 authors of scientific publications and several employees at the institutions in fact have been subject to investigation. Regarding individual persons, the Commission, in line with what is stated in Chapter 2, has applied a relatively high threshold for the circumstances that are to form the basis for criticism of individuals, namely gross and serious breaches of good research ethics perpetrated with intent or gross negligence.

The Commission has not found any grounds for believing that other individuals than Jon Sudbø have contributed to the fabrication of data or committed similar gross and serious breahes of regulations either intentionally or with gross negligence. However, Sudbø's supervisor and most important partner, Albrecht Reith, must suffer a certain criticism for lack of due care.

The fact that the threshold for criticism of individuals is so high, means that few individuals are subject to direct criticism by the Commission. The investigation has disclosed several less gross and serious cases of failing to comply with authorship criteria and the handling of patient data contrary to regulations which, per se, could have given grounds for criticism against more individuals. As mentioned in Chapter 2, however, such an investigation of less serious circumstances would have become disproportionately demanding. Less gross deviations are also serious to research, in particular if seen in connection (collective and cumulative errors). They are a threat to the quality of research and the population's trust in research. On this background, the Commission has chosen to identify less gross, but nevertheless serious criticizable circumstances, on a more general basis, without mentioning individual persons by name. As accounted for in the introduction, the Commission has chosen to concentrate its investigation on *gross* breaches of good research ethics, and therefore it does not have a sufficient basis for naming individuals as regards less serious, but nevertheless criticizable circumstances. Moreover, the errors concerned seem to have a certain general incidence, i.e. that the criticism is more related to systems rather than individuals since the deviations to a certain degree must have been known to and therefore apparently accepted by management. The Commission is of the opinion that identifying individuals in such a situation easily will give a distorted impression. As the Commission sees it, it is the duty of the institutions to ensure an appropriate training, organization and monitoring of the institution's activities, including the research activity. It must be an unconditional requirement that statutes,

regulations and work instructions are made known among the employees and that a certain regular monitoring that the rules are in fact complied with is maintained. In the Commission's opinion this would imply simple measures which would not be very cost demanding for the management and not very invasive to the researchers. It should be noted here that an evident improvement of these circumstances has occurred over the last years. The Commission will present more specific suggestions for development measures in Chapter 8.

Where criticism of institutions is concerned, the threshold for critical remarks is thus set considerably lower, both in relation to the gravity and the standard of proof of breaches. As regards the "system criticism" the Commission has elected to voice the impressions that it is left with, although the impressions are based on an incomplete and therefore more uncertain basis. The appointing bodies have, as opposed to individual persons, not been given the opportunity to respond to this criticism either. The Commission will nevertheless point at some systematic errors and flaws, because research institutions, and not only those directly involved, probably have a few things to learn from this case and the circumstances disclosed in its wake.

It is the Commission's hope that the institutional criticism will be perceived as constructive measures for improvement which may enhance the quality and trust in research in the long term.

7.2   Criticism of individual persons

7.2.1   Jon Sudbø

Based on the facts it has found to exist and accounted for in Chapter 4 and the assessments accounted for in Chapters 5 and 6, the Commission finds that Jon Sudbø has broken a series of rules. Most of these breaches have been committed with intent or gross negligence, and without doubt form the basis for criticism and the term scientific dishonesty. The breaches of rules must be seen in context. They occurred systematically from the end of the 1990's and up to the dishonesty was discovered in January 2006.

As accounted for in section 2.4.5, Sudbø has been allowed to read two draft reports with documentation annexed, among other things. He submitted a series of suggestions to the first draft, but refrained from commenting the revised draft he was subsequently sent. The Commission has considered Sudbø's suggestions and taken them into account to the extent it found grounds to do so. Apart from one admitted case of fabrication of data in connection with the Lancet article, and some minor admissions regarding two other articles, Sudbø is in all essentials uncomprehending to the fact that the Commission has found that the extent of scientific dishonesty is far more widespread.

In summary, this concerns the following circumstances:

- Manipulation and fabrication of data which form the basis for the PhD dissertation and which form the basis for 13 scientific publications which now must be retracted, see section 4.2-4.4.

- Manipulation and fabrication of data which form the basis for an article in Journal of Clinical Oncology 2005, see section 4.4.

- Manipulation and fabrication of data which form the basis for an article in The Lancet in 2005, see section 4.4.

- Failing to comply with the duty to submit to the Regional Ethical Committee and for a licence from the Data Inspectorate. Unlawful dealing with and access to sensitive patient data, i.e. lack of participants' consent and/or dispensation from the duty of secrecy from the Norwegian Board of Health (now: the Directorate for Health and Social Affairs), see section 4.2.4.

- Obvious erroneous information and misleading information in publications. For example, it is alleged that the histologic classification was made by four independent pathologists, that the ploidy analysis was performed by four observers, that patients had been included in an annual systemized follow-up project, that patients were included in smoking cessation projects and the like, see in particular section 4.2.

- Intentional misrepresentation in connection with an application for financial support from the National Cancer Institute (NCI), see in particular section 5.3.

- Breach of good scientific practice for including and excluding authors in publications. Co-authors have been abused and misled, see in particular sections 5.2 and 5.3.

- Misleading of sponsors and his employer and others who have provided financial support to Jon Sudbø and his research, including in particular the Cancer Society, Health and Rehabilitation and the Radiumhospitalet, see Chapter 4 to 6.

- Misleading of his own PhD candidate who was given data material which partly was based on fabricated data from Jon Sudbø as the main supervisor. The consequence of this is that the candidate has spent several years on a project which recently was completed, but which now must be retracted. The candidate is probably put back at least two years in time in relation to a possible presentation of a doctoral thesis, see in particular section 4.2.

- Harming the reputation of research. The Commission here refers to the risk of harm to the trust in research in general caused by the dishonest activities, see section 6.1.

- Jeopardizing patients' safety. Dishonesty in connection with this type of medical research is particularly serious because it entails an obvious danger that the invalid research results will mislead patients, health staff and researchers. This risk must have been obvious to Jon Sudbø. Although the Commission has not seen it as its task to pursue this, it is reasonable to assume that the dishonest

research has had unfortunate and harmful consequences for the diagnosis and treatment of individual patients, see in more detail section 6.2.

7.2.2 Albrecht Reith

The Commission has found grounds for criticism against Professor Albrecht Reith MD at the Rikshospitalet/Radiumhospitalet MC in relation to certain circumstances. This is primarily related to Reith's role as Jon Sudbø's PhD supervisor and subsequent primary collaboration partner. In the Commission's opinion, generally a supervisor is responsible for guiding and monitoring his/her research fellow. The Commission has found that Reith to a certain extent has failed as a supervisor by inadequatefollowing up. This has been a contributing factor to Jon Sudbø having been able to act in contravention of good scientific practice.

The Commission has not found grounds to state that these errors were committed with intent or gross negligence or that Reith is guilty of so-called scientific dishonesty. The Commission has found it to be proven that Reith in some cases should have acted differently, and that he is to blame for not doing so. Thus, it is a question of ordinary negligence. It seems as if Reith had boundless trust in Sudbø. The Commission finds reason to comment that Reith has been highly cooperative in connection with the investigation.

Reith has been allowed to read two drafts of the report and he has submitted comments to certain factual matters. The Commission has considered and taken into account these comments as far as the Commission has found grounds for doing so. Reith has not raised objections to the criticism stated.

In summary, the Commission would in particular emphasize the following circumstances as criticizable:

- Insufficient supervision and due care in relation to obtaining necessary advance evaluations and permissions from the Regional Committee for Medical Research Ethics, the Data Inspectorate and the Board of Health (now: the Directorate for Health and Social Affairs).
- Insufficient supervision and due care in relation to the handling of patient information subject to secrecy.
- Insufficient supervision and due care in relation to the practicing of general principles for authorship. Reference is here made to the fact that several authors brought up Jon Sudbø's unusual and unlawful practicing of authorship directly with Reith, who apparently did not see reason to follow up and correct this practice. On the contrary, it is the Commission's impression that Reith protected Sudbø and prevented a further investigation of accusations of unacceptable practice.
- Insufficient due care relating to several publications, in which a series of errors appear. As main supervisor and last author, Reith should have reacted to at least some of these errors. This concerns i.a. the

statement that four independent pathologists had reclassified the entire raw material for the oral cancer project, and that four independent observers had evaluated the ploidy analyses.

7.3 Criticism of institutions

7.3.1 The Rikshospitalet – Radiumhospitalet MC

The Commission has found reason to criticize the Rikshospitalet/Radiumhospitalet MC represented by its general management regarding a series of circumstances. The Commission's basis is that Sudbø during his entire scientific career primarily was associated with the Radiumhospitalet, which then had the overall everyday responsibility for his research which was performed under the auspices of the Radiumhospitalet, cf section 4.2. It should be noted that prior to January 1, 2002, the State represented by the Ministry of Health, was responsible for the Radiumhospitalet. As from and including January 1, 2002, the Radiumhospitalet became a separate medical center and thus a separate legal entity. As from and including January 1, 2005, the Rikshospitalet MC and the Radiumhospitalet MC were merged into one medical center and legal entity: The Rikshospitalet – Radiumhospitalet MC. The medical center has not been given the opportunity to respond to this criticism, cf section 2.4.6.

In introduction, the Commission would emphasize that this criticism is not based on comprehensive investigations of the management, but rather on more or less clear impressions which the Commission is left with after investigating a specific personnel matter. The Commission nevertheless finds reason to mention these impressions.

When a research institution, which the MC is, makes provisions for research at the institution, it must be prepared to carry the full responsibility for the individual researcher and the relevant research project, regardless of whether others also have an independent responsibility. Patients and others who relate to the MC, including collaborating institutions, must be able to expect that researchers at the MC work on behalf of the same MC, and that the MC has the overall responsibility.

The medical center must therefore suffer criticism for what appears as inadequate training, management and control of Jon Sudbø's and other employees' research activities at the institution. This has probably been a contributing factor to the dishonest research being able to take place and be carried on for such a relatively long time.

Several researchers at the institution have described situations from earlier periods which in the Commission's opinion indicate a disturbing lack of awareness of the prevailing rules for good research practice. This applies in particular to rules on secrecy, protection of personal data, authorship and advance assessments of research projects which are the rules which have been particularly relevant in this case. Furthermore, the distribution of responsibility regarding the institution's research have been unclear

and too much of the activity has been left to the individual researcher. Unacceptable matters which in fact were pointed out, and which could have brought the dishonest research to light, were not followed up and managed in a satisfactory way.

Here should be noted that there is no lack of good intentions. The management has had a very clear attitude as regards its own responsibility and high expectations to its own employees. In conversations with the Commission, the management referred to internal work instructions and other measures as for example the so-called Protocol Committee and coordinating office for research, which is meant to contribute to the implementation of rules and regulations and enhanced research practice. The management also had a very clear attitude regarding expectations that the employees comply with all statutes, regulations and work instructions, including the Helsinki Declaration as well as the Vancouver Rules. However, the Commission is left with an impression that these measures have not been followed up well enough. By this, an attitude among the employees has been allowed to develop to the effect that after all it did not matter so much with for example the duty of secrecy, recommendations from the Regional Committee for Medical Research Ethics and practicing of the authorship criteria. Several people stated to the Commission that the Vancouver Rules are not binding for them – they are only guidelines, whereas the management stated that they evidently enough are binding on their employees. The management must assume the responsibility for this discrepancy and confusion.

The Commission has the impression that circumstances have become/are becoming better. Insufficient follow up of research by management was hardly an unusual phenomenon at some Norwegian research institutions some years ago. Traditionally, researchers have worked very freely, both at universities and Norwegian hospitals. Clinicians have been encouraged to carry on research, but sufficient awareness related to the different roles and partly different requirements put to them has probably not been sufficient, neither on the part of researchers nor management. Insufficient and failing routines, and an insufficient system for notifying irregularities, have been an unfortunate combination for the Radiumhospitalet. The medical research community is thus in a transition phase as regards the organization and formalities relating to medical research. The Commission believes that this specific case has been an eye-opener for this as well as for other research institutions, and will probably contribute to speeding up this development process.

The Commission nevertheless finds reason to maintain the criticism of the Rikshospitalet/Radiumhospitalet MC. The criticizable circumstances can be summarized as:

- Insufficient advance control and organization of Sudbø's PhD project, including specification of distribution of responsibility.
- Insufficient training and consciousness-raising of Sudbø and other employees about the rules for handling patient material, advance assessment of research projects and authorship.

- Insufficient management and routines for discovering and handling deviations from internal instructions, etc.

7.3.2   The University of Oslo – The Odontology

The Commission finds reason to level a certain criticism against the University of Oslo, the Faculty of Odontology, for delivering patient material and data from the Odontology to Jon Sudbø and Albrecht Reith without the existence of participant's consents or dispensations from the duty of secrecy as required at that time, cf section 4.2.7, cf section 4.2.4. The delivery of data therefore appears as a breach of the regulations in force at that time. The University of Oslo has not been given the opportunity to respond to this criticism, cf section 2.4.6.

In this context is noted that Jon Sudbø was employed in a 20% position only at the University of Oslo from May 2, 2005 until the beginning of 2006 and also that the University during this period had a more secondary general responsibility for Jon Sudbø's research activities, compared with the Rikshospitalet/Radiumhospitalet MC.

7.3.3   The University of Bergen – Gade's Institute

The Commission has found reason to level a certain criticism against the University of Bergen, Department of Pathology, "Gade's Institute", for not having made sure that Jon Sudbø and Albrecht Reith had obtained consent from the patients or dispensation from the duty of secrecy when patient material and data were delivered to them, cf section 4.2.7, cf section 4.2.4. The delivery of data thus appears as a breach of the regulations in force at that time. The University of Bergen has not commented on the Commission's draft of criticism.

7.3.4   The Cancer Registry

The Commission considered whether there was reason to level a certain criticism against the Cancer Registry for not having made sure that Jon Sudbø and Albrecht Reith had obtained consent from the patients or dispensation from the duty of secrecy when cancer registry data connected to their patient data in 1996 were delivered to them, cf section 4.2.7, cf section 4.2.4. The framework concession of December 9, 1985, item 4.3 no. 2, third dash line, states that the delivery of information for research purposes was conditional on observance of the rules on secrecy, where appropriate after dispensation. No dispensation for the relevant delivery was granted.

The Cancer Registry was notified that the Commission considered to level a certain criticism on this basis and made use of its right to comment on an earlier draft of section 7.3.4 of the report. In a letter to the Commission dated June 20, 2006, the Cancer Registry asserted that the draft criticism is based on an erroneous conception of the Cancer Registry's different roles. The Cancer Registry asserts that it understood Jon Sudbø's request of February 20, 1996, as a routine request for follow-up data for patients at the

Radiumhospitalet, Department of Pathology, which did not require advance permission, such as for example dispensation from the duty of secrecy, see in more detail section 4.2.7.

The Commission has considered the Cancer Registry's comments and compared them with the information given in Jon Sudbø's letter of February 20, 1996 to the Cancer Registry, quoted in section 4.2.7, and other information in the case. On that background, the Commission finds that it appears from the letter that it is a question of a research project, something which the Cancer Registry should have understood. The delivery of data then appears as a breach of the licence conditions.

7.4   General remarks

As mentioned, the Commission has investigated all 60 coauthors. The Commission has reviewed the role of the individual coauthor to see if anyone may be suspected of having participated in the fabrication of research data or other gross breaches of good scientific practice. The Commission has found that there are no reasons to believe that other persons than Jon Sudbø, either intentionally or with gross negligence, have contributed to the fabrication of data or committed similar gross and serious breaches of good scientific practice.

For general deterrence reasons, among other things, the Commission has nevertheless found reason to point out certain criticizable circumstances on a more general basis. The Commission has difficulty in understanding that the breaches of good scientific practice which have been discovered, probably have been ongoing for such a comparatively long period without anyone discovering it. In Chapter 5, the Commission touched upon how this may have happened. Obviously, Jon Sudbø had the main responsibility for the articles of which he was the first author. He had full control over the raw data and the analyses made. Moreover, he had full control over who was a coauthor and who was not. By this, he was able to distribute the work such that the different contributors/coauthors did not have much access to the other contributors/coauthors' actual tasks and contributions. By this distribution of tasks, the coauthors have been deceived by only having been involved to a rather restricted degree. At the same time, they were involved in such a way that they "nevertheless" accepted the authorship. Admittedly, several of them expressed doubt about whether they should take part as coauthor, and brought this up with Jon Sudbø and Albrecht Reith. Some even pointed out that the coauthor practice followed by Jon Sudbø was unacceptable, and made this clear to Jon Sudbø and Albrecht Reith. They were then told that this view was taken note of and that it should not happen again. No improvement took place, however. These authors were instead excluded from further collaboration.

In retrospect it is obvious that many persons ought to have become suspicious, reacted more strongly and investigated matters more closely. In this context, several persons have had occasion to notify the

management of the institution. There were also several persons in the medical community who were suspicious and skeptical to Jon Sudbø's research and pointed this out to Jon Sudbø as well as Albrecht Reith and the management at the Radiumhospitalet. However, no proper routines for notification existed, and criticism against Jon Sudbø was brushed aside, explained away and petered out. In fact, several persons have stated to the Commission that they did not want to end up in a whistleblower position, and for that reason refrained from making further investigations. They maintain that ending up in the position of a whistleblower would be a great personal burden for them, in particular when a researcher who had a sort of status as the community's "favorite son" was involved.

The reason that the Commission has not chosen to make a more detailed and thorough investigation of individual persons for less serious breaches of good scientific practice is also that the Commission understands that the practicing of authorship criteria is hardly unique to this case.

Several coauthors have been listed without their knowledge. Some of them became aware of such listings after publication and brought up this unacceptable practice with Jon Sudbø without any ensuing consequences. These circumstances could and should have been reported to the management, providing the management with an opportunity to take action on a more principal basis. A general characteristic seems to be that many of the coauthors did not have a very conscious relationship to the responsibility inherent in being listed as a coauthor of a scientific publication. In other words, they have taken this role and responsibility too lightly.

The media has devoted much attention to the fact that Jon Sudbø's co-habitant, Wanja Kildal, and his brother, Asle Sudbø, were coauthors in several publications. For that reason the Commission will, in conclusion, remark that both of them collaborated with the Commission and contributed to illuminating the case. Having account to the extensive media focus, with its inherent suspicions directed against these two persons, the Commission finds reason to underline that the investigation has not disclosed any grounds for believing that any of them have been guilty in or contributed to scientific dishonesty.

8. Recommendations

8.1. The institutions

As accounted for in Chapter 5, the Commission is of the opinion that the breaches of good scientific practice which have been disclosed, can hardly be explained by a lack of research-ethical rules and principles. Nor can they be explained solely by reference to a single dishonest researcher. The interaction of a series of unfortunate circumstances has played a role in this case. On this background, the Commission has elected to emphasize the research communities, and in particular the research institution's, joint responsibility to promote honest and ethically proper research of a good quality.

The Commission considers it to be outside its terms of reference to submit detailed suggestions as to how improvements of internal institutional control routines and the organization of research should be implemented. However, the Commission will point out some circumstances of a more general nature, which may prove effective and relatively simple to implement:

Institutional implementation of the prevailing set of rules

Research institutions must to a larger extent make all researchers and supervisors aware of the prevailing rules and the liability attached to to breaches of the rules. Ensuring that applicable statutes and rules and regulations are complied with and enforced is to a large degree an institutional system and management responsibility, where, in the Commission's opinion, there is an evident potential for improvement. For example, the responsible research institution must have a satisfactory overview of and exercise satisfactory control over all research projects taking place at, or under the auspices of, such institution, without it necessarily being in conflict with academic freedom. The need for preventive work, and the research institutions' responsibility for development of environments which take care of lifelong learning within the scope of research ethics and good research practice, is also emphasized in the preparatory works to the newly enacted Research Ethics Act (not yet in force). For example, the institutions could have been more explicit regarding whether and to what extent the Vancouver Rules are to be considered as binding for their employees. The Commission would also here refer to the simple but good advice to be found in *Guidelines for the implementation of research projects related to medicine and health* (Annex 5).

Handling of data and ethical evaluation of projects

As a minimum, the institutions should have routines to ensure that necessary permits for research involving people, human biological material, personal data information and/or animals are in place when research projects are initiated. In addition, the universities should require documentation that these are in place when PhD dissertations are submitted, for example.

Filing of research data

The research institutions should create systems taking care of the filing and storing of research data, as, i.a., the Research Council requires when funds are granted.

The contents of the supervisor role

The content of the supervisor role must be made more explicit, and a more precise responsibility should be imposed on the supervisor to ensure that research-ethical factors are understood.

Notification of errors and flaws

Better arrangements for notification of errors and flaws should also be implemented, for example by the creation of a researcher ombudsman, as suggested by some.

8.2 The journals

In the Commission's opinion, this case has disclosed certain weaknesses in the routines practiced in connection with the publication of medical research articles. The Commission believes that, in order to contribute to the proper compliance with prevailing rules, medical journals should introduce and practice a system in which all coauthors are made part of the communication with the journals. They should be sent letters informing them that they have been stated as coauthors, and they should individually submit their confirmations of this directly to the respective editors. At the same time, the authors should submit a written account of what their intellectual contribution to the work consists of, which is something some journals require already. Likewise, the Commission believes that it would be reasonable that all coauthors are sent review statements. In this way the individual author's awareness of his/her responsibility would be strengthened, and one would also avoid that researchers are listed as authors without any knowledge of this fact.

If such principles had been applied to the Lancet article, it is, in the Commission's opinion, fairly likely that the research fraud would have been discovered already prior to publication, in particular considering fairly negative statements and critical questions by one of the professional colleagues who

reviewed the articles. Corresponding principles should apply to contributors who are listed under acknowledgements.

With the use of modern electronic communications, these suggestions for changed routines ought not to imply substantial administrative or financial burdens, in the Commission's opinion. In order to alleviate the administrative challenges, one should therefore require that the submission of manuscripts be accompanied by a list of the email addresses of all authors/coauthors and contributors.

Furthermore, the Commission has considered whether the system of "fast-track publishing" which is practiced by some journals, may have unfortunate effects, in the sense that there is too little time between the evaluation by professional colleagues and publication for professional objections to the work to form part of the procedure. The Commission here confines itself to pointing at the problem. Even though the persons who are employed as professional colleagues in a "fast track" review system possess solid professional weight and integrity, one can nevertheless not entirely disregard that the time pressure inherent in the system itself generates an unnecessary risk that errors are not discovered.

8.3   The Commission's concluding remark

In conclusion, the Commission will remark that although this case has been very serious and tragic for individuals, institutions and Norwegian research more generally, there appears to be a remarkable will internally in the research community to turn the case around to something positive – something everyone can learn from. Frequent contributions to newspapers, debates and seminars have shown an already great involvement in many research environments. The Commission will support such a way of thinking, and at the same time caution against sweeping this extraordinary case under the carpet – because it *is* extraordinary – and just continue as before.

On the contrary, this case provides an opportunity for a thorough discussion of different sides of the norms related to good research practice. The research community must make an all-out effort to make plain research's traditional ideals of honesty, thoroughness, trustworthiness and openness. And this must be made visible to the general public so that the population's trust in Norwegian research is maintained and reinforced.

Annexes

Annex 1: Jon Sudbø's publication list[48]

1: Sudbo J, Lee JJ, Lippman SM, Mork J, Sagen S, Flatner N, Ristimaki A, Sudbo A, Mao L, Zhou X, Kildal W, Evensen JF, Reith A, Dannenberg AJ. Non-steroidal anti-inflammatory drugs and the risk of oral cancer: a nested case-control study. Lancet. 2005 Oct 15-21;366(9494):1359-66.

2: Sudbo J. Novel management of oral cancer: a paradigm of predictive oncology. Clin Med Res. 2004 Nov;2(4):233-42. Review.

3: Kildal W, Risberg B, Abeler VM, Kristensen GB, Sudbo J, Nesland JM, Danielsen HE. betacatenin _expression, DNA ploidy and clinicopathological features in ovarian cancer: a study in 253 patients. Eur J Cancer. 2005 May;41(8):1127-34. Epub 2005 Apr 14.

4: Lilleby W, Sudbo J, Fossa SD. [Biology and treatment options during the development of prostate cancer] Tidsskr Nor Laegeforen. 2005 Mar 3;125(5):571-4. Review. Norwegian.

5: Sudbo J, Samuelsson R, Risberg B, Heistein S, Nyhus C, Samuelsson M, Puntervold R, Sigstad E, Davidson B, Reith A, Berner A. Risk markers of oral cancer in clinically normal mucosa as an aid in smoking cessation counseling. J Clin Oncol. 2005 Mar 20;23(9):1927-33.

6: Sudbo J, Reith A. The evolution of predictive oncology and molecular-based therapy for oral cancer prevention. Int J Cancer. 2005 Jun 20;115(3):339-45. Review.

7: Lippman SM, Sudbo J, Hong WK. Oral cancer prevention and the evolution of moleculartargeted drug development. J Clin Oncol. 2005 Jan 10;23(2):346-56. Review.

8: Kildal W, Kaern J, Kraggerud SM, Abeler VM, Sudbo J, Trope CG, Lothe RA, Danielsen HE. Evaluation of genomic changes in a large series of malignant ovarian germ cell tumors--relation to clinicopathologic variables. Cancer Genet Cytogenet. 2004 Nov;155(1):25-32.

9: Sudbo J, Lippman SM, Lee JJ, Mao L, Kildal W, Sudbo A, Sagen S, Bryne M, El-Naggar A, Risberg B, Evensen JF, Reith A. The influence of resection and aneuploidy on mortality in oral leukoplakia. N Engl J Med. 2004 Apr 1;350(14):1405-13.

10: Sudbo J, Reith A, Florenes VA, Nesland JM, Ristimaki A, Bryne M. COX-2 _expression in striated muscle under physiological conditions.
Oral Dis. 2003 Nov;9(6):313-6.

11: Sudbo J. Non-invasive early diagnosis of oral cavity malignancies.
Anal Cell Pathol. 2003;25(4):157-8.

12: Sudbo J, Bryne M, Mao L, Lotan R, Reith A, Kildal W, Davidson B, Soland TM, Lippman SM. Molecular based treatment of oral cancer.
Oral Oncol. 2003 Dec;39(8):749-58. Review.

13: Sudbo J. Protection of research subjects. N Engl J Med. 2003 Jul 10;349(2):188-92; author reply 188-92.

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[48] Taken from www.pubmed.gov Januaary 2006. Not exhaustive.

14: Sudbo J. [Chemoprevention of oral cancer] Tidsskr Nor Laegeforen. 2003 May 29;123(11):1518-21. Review. Norwegian.

15: Sudbo J. [Chemoprevention: treatment of persons at high risk of cancer] Tidsskr Nor Laegeforen. 2003 May 29;123(11):1514-7. Review. Norwegian.

16: Sudbo J, Ristimaki A, Sondresen JE, Kildal W, Boysen M, Koppang HS, Reith A, Risberg B, Nesland JM, Bryne M. Cyclooxygenase-2 (COX-2) expression in high-risk premalignant oral lesions. Oral Oncol. 2003 Jul;39(5):497-505.

17: Scully C, Sudbo J, Speight PM. Progress in determining the malignant potential of oral lesions. J Oral Pathol Med. 2003 May;32(5):251-6. Review.

18: Sudbo J, Reith A. When is an oral leukoplakia premalignant?
Oral Oncol. 2002 Dec;38(8):813-4; author reply 811-2. No abstract available.

19: Sudbo J, Reith A. Which putatively pre-malignant oral lesions become oral cancers? Clinical relevance of early targeting of high-risk individuals.
J Oral Pathol Med. 2003 Feb;32(2):63-70. Review.

20: Reith A, Sudbo J. Impact of genomic instability in risk assessment and chemoprevention of oral premalignancies. Int J Cancer. 2002 Sep 20;101(3):205-9. Review.

21: Sudbo J. [Adverse effects of COX-2 inhibitors] Tidsskr Nor Laegeforen. 2002 Jan 10;122(1):102-3. Norwegian.

22: Sudbo J. [DNA ploidy analysis--a possibility for early identification of patient with risk of oral cancer] Lakartidningen. 2001 Nov 7;98(45):4980-4. Review. Norwegian.

23: Sudbo J. Pathology in disgrace? J Pathol. 2002 Feb;196(2):244-5. No abstract available.

24: Sudbo J, Kildal W, Johannessen AC, Koppang HS, Sudbo A, Danielsen HE, Risberg B, Reith A. Gross genomic aberrations in precancers: clinical implications of a long-term follow-up study in oral erythroplakias. J Clin Oncol. 2002 Jan 15;20(2):456-62.

25: Sudbo J, Warloe T, Aamdal S, Reith A, Bryne M. [Diagnosis and treatment of oral precancerous lesions] Tidsskr Nor Laegeforen. 2001 Oct 30;121(26):3066-71. Norwegian.

26: Sudbo J, Ried T, Bryne M, Kildal W, Danielsen H, Reith A.
Abnormal DNA content predicts the occurrence of carcinomas in non-dysplastic oral white patches. Oral Oncol. 2001 Oct;37(7):558-65.

27: Sudbo J. Human papillomavirus infection as a risk factor for squamous-cell carcinoma of the head and neck. N Engl J Med. 2001 Aug 2;345(5):376-7.

28: Sudbo J, Bryne M, Johannessen AC, Kildal W, Danielsen HE, Reith A. Comparison of histological grading and large-scale genomic status (DNA ploidy) as prognostic tools in oral dysplasia. J Pathol. 2001 Jul;194(3):303-10.

29: Sudbo J, Reith A, Lingjaerde OC. Gene-_expression profiles in hereditary breast cancer. N Engl J Med. 2001 Jun 28;344(26):2029. No abstract available.

30: Sudbo J, Kildal W, Risberg B, Koppang HS, Danielsen HE, Reith A. DNA content as a prognostic marker in patients with oral leukoplakia. N Engl J Med. 2001 Apr 26;344(17):1270-8.

31: Sudbo J, Marcelpoil R, Reith A. New algorithms based on the Voronoi Diagram applied in a pilot study on normal mucosa and carcinomas. Anal Cell Pathol. 2000;21(2):71-86.

32: Sudbo J, Marcelpoil R, Reith A. Caveats: numerical requirements in graph theory based quantitation of tissue architecture. Anal Cell Pathol. 2000;21(2):59-69.

33: Sudbo J, Bankfalvi A, Bryne M, Marcelpoil R, Boysen M, Piffko J, Hemmer J, Kraft K, Reith A. Prognostic value of graph theory-based tissue architecture analysis in carcinomas of the tongue. Lab Invest. 2000 Dec;80(12):1881-9.

34: Danielsen HE, Kildal W, Sudbo J. [Digital image analysis in pathology--exemplified in prostatic cancer] Tidsskr Nor Laegeforen. 2000 Feb 10;120(4):479-88. Review. Norwegian.

35: Bryne M, Boysen M, Alfsen CG, Abeler VM, Sudbo J, Nesland JM, Kristensen GB, Piffko J, Bankfalvi A. The invasive front of carcinomas. The most important area for tumour prognosis? Anticancer Res. 1998 Nov-Dec;18(6B):4757-64. Review.

36: Zhang Z, Suo Z, Sudbo J, Holm R, Boysen M, Reith A. Diagnostic implications of p53 protein reactivity in nasal mucosa of nickel workers.
Anal Quant Cytol Histol. 1997 Aug;19(4):345-50.

37: Xie X, Clausen OP, Sudbo J, Boysen M. Diagnostic and prognostic value of nucleolar organizer regions in normal epithelium, dysplasia, and squamous cell carcinoma of the oral cavity. Cancer. 1997 Jun 1;79(11):2200-8.

38: Bjaalie JG, Sudbo J, Brodal P. Corticopontine terminal fibres form small scale clusters and large scale lamellae in the cat. Neuroreport. 1997 May 6;8(7):1651-5.

Annex 2: Evolvement of the authorship criteria

1997 International Committee of Medical Journal Editors. Uniform requirements for manuscripts submitted to biomedical journals. N Engl J Med. 1997 Jan 23;336(4):309-15.		**Authorship** All persons designated as authors should qualify for authorship. **Each author should have participated sufficiently in the work to take public responsibility for the content.** **Authorship credit should be based only on substantial contributions to** **(a)** conception and design, **or** analysis and interpretation of data; and to **(b)** drafting the article or revising it critically for important intellectual content; and on **(c)** final approval of the version to be published. **Conditions (a), (b), and (c) must all be met.** Participation solely in the acquisition of funding or the collection of data does not justify authorship. General supervision of the research group is not sufficient for authorship. **Any part of an article critical to its main conclusions must be the responsibility of at least one author.** Editors may ask authors to describe what each contributed; this information may be published. Increasingly, multicenter trials are attributed to a corporate author. All members of the group who are named as authors, either in the authorship position below the title or in a footnote, should fully meet the above criteria for authorship. Group members who do not meet these criteria should be listed, with their permission, in the Acknowledgments or in an appendix (see Acknowledgments). The order of authorship should be a joint decision of the coauthors. Because the order is assigned in different ways, its meaning cannot be inferred accurately unless it is stated by the authors**.** **Authors may wish to explain the order of authorship in a footnote.** In deciding on the order, authors should be aware that many journals limit the number of authors listed in the table of contents and that the National Library of Medicine lists in MEDLINE only the first 24 plus the last author when there are more than 25 authors.
2003 Davidoff F, Godlee F, Hoey Glass R, Overbeke J, Utiger R, Nicholls MG, Horton R, Nylenna M, Hojgaard L, Kotzin S; International Committee of Medical Journal Editors. Uniform requirements for manuscripts submitted to biomedical journals. J Am Osteopath Assoc. 2003 Mar;103(3):137-49.		**Authorship** All persons designated as authors should qualify for authorship, and all those who qualify should be listed. **Each author should have participated sufficiently in the work to take public responsibility for appropriate portions of the content. One or more authors should take responsibility for the integrity of the work as a whole, from inception to published article.** Authorship credit should be based only on (1) substantial contributions to conception and design, **or** acquisition of data, **or** analysis and interpretation of data; (2) drafting the article or revising it critically for important intellectual content; and (3) final approval of the version to be published. **Conditions 1, 2, and 3 must all be met.** Acquisition of funding, the collection of data, or general supervision of the research group, by themselves, do not justify authorship. Authors should provide a description of what each contributed, and editors should publish that information. All others who contributed to the work who are not authors should be named in the Acknowledgments, and what they did should be described

		(see Acknowledgments, page 140). Increasingly, authorship of multicenter trials is attributed to a group. All members of the group who are named as authors should fully meet the above criteria for authorship. Group members who do not meet these criteria should be listed, with their permission, in the Acknowledgments or in an appendix (see Acknowledgments, page 140). **The order of authorship on the byline should be a joint decision of the coauthors. Authors should be prepared to explain the order in which authors are listed.**

2006 Uniform Requirements Manuscripts Submitted Biomedical Journals: Writing and Editing for Biomedical Publication. Updated February 2006 www.icmje.org	**II.A Authorship and Contributorship** **II.A.1. Byline Authors** **An "author" is generally considered to be someone who has made substantive intellectual contributions to a published study, and biomedical authorship continues to have important academic, social, and financial implications.** (1) In the past, readers were rarely provided with information about contributions to studies from those listed as authors and in acknowledgments. (2) Some journals now request and publish information about the contributions of each person named as having participated in a submitted study, at least for original research. Editors are strongly encouraged to develop and implement a contributorship policy, as well as a policy on identifying who is responsible for the integrity of the work as a whole. While contributorship and guarantorship policies obviously remove much of the ambiguity surrounding contributions, it leaves unresolved the question of the quantity and quality of contribution that qualify for authorship. The International Committee of Medical Journal Editors has recommended the following criteria for authorship; these criteria are still appropriate for those journals that distinguish authors from other contributors. • **Authorship credit should be based on** **1)** substantial contributions to conception and design, **or** acquisition of data, **or** analysis and interpretation of data; **2)** drafting the article or revising it critically for important intellectual content; and **3)** final approval of the version to be published. **Authors should meet conditions 1, 2, and 3.** • When a large, multi-center group has conducted the work, the group should identify the individuals who accept direct responsibility for the manuscript (3). These individuals should fully meet the criteria for authorship defined above and editors will ask these individuals to complete journal-specific author and conflict of interest disclosure forms. When submitting a group author manuscript, the corresponding author should clearly indicate the preferred citation and should clearly identify all individual authors as well as the group name. Journals will generally list other members of the group in the acknowledgements. The National Library of Medicine indexes the group name and the names of individuals the group has identified as being directly responsible for the manuscript. • Acquisition of funding, collection of data, or general supervision of the research group, alone, does not justify authorship. • All persons designated as authors should qualify for authorship, and all those who qualify should be listed. • **Each author should have participated sufficiently in the work to take public responsibility for appropriate portions of the content. Some journals now also request that one or more authors, referred to as "guarantors," be identified as the persons who take responsibility for the integrity of the work as a whole, from inception to published article, and publish that information.** Increasingly, authorship of multi-center trials is attributed to a group. All members of the group who are named as authors should fully meet the above criteria for authorship. The order of authorship on the byline should be a joint decision of the coauthors. **Authors should be prepared to explain the order in which authors are listed.**

1997 International Committee of Medical Journal Editors. Uniform requirements for manuscripts submitted to biomedical journals. N Engl J Med. 1997 Jan 23;336(4):309-15. .		**Acknowledgments** At an appropriate place in the article (the title-page footnote or an appendix to the text; see the journal's requirements) one or more statements should specify (a) contributions that need acknowledging but do not justify authorship, such as general support by a departmental chair; (b) acknowledgments of technical help; (c) acknowledgments of financial and material support, which should specify the nature of the support; and (d) relationships that may pose a conflict of interest. Persons who have contributed intellectually to the paper but whose contributions do not justify authorship may be named and their function or contribution described — for example, "scientific advisor," "critical review of study proposal," "data collection," or "participation in clinical trial." Such persons must have given their permission to be named. **Authors are responsible for obtaining written permission from persons acknowledged by name, because readers may infer their endorsement of the data and conclusions.** Technical help should be acknowledged in a paragraph separate from those acknowledging other contributions
2003 Davidoff F, Godlee F, Hoey J, Glass R, Overbeke J, Utiger R, Nicholls MG, Horton R, Nylenna M, Hojgaard L, Kotzin S; International Committee of Medical Journal Editors. Uniform requirements for manuscripts submitted to biomedical journals. J Am Osteopath Assoc. 2003 Mar;103(3):137-49.		**Acknowledgments** List all contributors who do not meet the criteria for authorship, such as a person who provided purely technical help, writing assistance, or a department chair who provided only general support. Financial and material support should also be acknowledged. Groups of persons who have contributed materially to the paper but whose contributions do not justify authorship may be listed under a heading such as "clinical investigators" or "participating investigators," and their function or contribution should be described-for example, "served as scientific advisors," "critically reviewed the study proposal," "collected data," or "provided and cared for study patients." **Because readers may infer their endorsement of the data and conclusions, all persons must have given written permission to be acknowledged.**

2006 Uniform Requirements for Manuscripts Submitted to Biomedical Journals: Writing and Editing for Biomedical Publication Updated February 2006 www.icmje.org	**II.A.2. Contributors Listed in Acknowledgments** All contributors who do not meet the criteria for authorship should be listed in an acknowledgments section. Examples of those who might be acknowledged include a person who provided purely technical help, writing assistance, or a department chair who provided only general support. Editors should ask authors to disclose whether they had writing assistance and to identify the entity that paid for this assistance. Financial and material support should also be acknowledged. Groups of persons who have contributed materially to the paper but whose contributions do not justify authorship may be listed under a heading such as "clinical investigators" or "participating investigators," and their function or contribution should be described—for example, "served as scientific advisors," "critically reviewed the study proposal," "collected data," or "provided and cared for study patients." **Because readers may infer their endorsement of the data and conclusions, all persons must give written permission to be acknowledged.**

Annex 3: Files and lists which the Commission has used in the investigation of the articles in New England Journal of Medicine 2001 and 2004

File name	Records	Note	Source/year[49]
Updated rawdata (Excel)	150 records	First file sent to the US for New England Journal of Medicine 2004 Does not have a link key	JJL 2006
Sudbø8 (Excel)	150 records	Last file sent to the US for New England Journal of Medicine 2004 Does not have a link key	JJL 2006
Reith paper list (rawdata oral mucosa)	150 records	Identical to Sudbø8 on date variables – contains also block numbers on 142 records which enables linkage	AR 2006
L29 (Excel)	147 records (block numbers)	Contains the same block number as Reith paper list. The heading of the list is "Date: April 15, 1998 Main record Jnr L29 Project Oral Mucosa Paraffin Blocks from Haukeland"	Radiumhospitalet
L31 (Excel)	106 records (block numbers)	Date: April 1998 Main record Jnr:L31 Project Oral Mucosa Paraffin Blocks from Haukeland. Cut 50umxn. Method: monolayer presentation. Protease 60min, Hydrolysis 60min	Sent from HD 2006
L34 (Excel)	69 records (block numbers)	Date: March 8, 1999 Main record Jnr:L34 Material: Dysplasia from Fac. of Odontology. Received blocks, cut 50um + 3 uncolored biopsy specimens Method: Monolayer Feulgen colored + color HE of 1 uncolored cut Protease 0.5mg/ml for 60 min 37C. 5N HCl 60min Basic Fuchsin 2 hours NB! Acc. to agreement with Jon this protocol is excluded. Samples in this series is given L31 October 23, 2001 Tidied in biopsy specimens and blocks. Biopsy specimens not changed to L31. Consequently filed under L34. Project responsible: Jon Sudbø Sign.: Date:	Sent from HD 2006
L47 (Excel)	64 records (block numbers)	Date: March 24, 2000 Main record Jnr:L47 Material: Normal mucosa Method: monolayer from paraffin-imbedded material 1. One HE cut if little material, only monolayer to be made 2. nx 50 um biopsy specimens 3. One HE2 cut Project responsible: Jon Sudbø Sign.: Date: December 2000. Project completed delivered to Jon Date: March 24, 2000 Main journal	Sent from RP 2006

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[49] JJL=J.Jack Lee, AR= Albrecht Reith, JS= Jon Sudbø, HD=Håvard Danielsen, RP=Ruth Punthervold

Ploidy list (Excel)	393 lines in the Excel sheet	File name: All Original blocks and HE biopsy specimens linked to ploidy prep and the L29 series.xls Contains a link between ploidy preparation and block number and link to the L29 series	Produced by RP 2006
Dnr-js2 (Excel)	226 persons – 61 Lacks p.id. no.	File delivered from the Cancer Registry to Sudbø in 1996	The Cancer Registry 1996
X diagnoses (SPSS)	311 records for 195 persons	All at Dnr-js2 with cancer diagnosis per 2006 (195 of 226 persons)	The Cancer Registry 2006
X persons (SPSS)	226 persons	All at Dnr-js2 supplemented by p.id.no. and date of death per 2006	The Cancer Registry 2006
Gade	590 records 162 persons	All referring letters from Gade concerning persons from Dnr-js2	The Cancer Registry 2006
The Odontology, Oslo	132 records 62 persons	All referring letters from the Odontology, Oslo, concerning persons from Dnr-js2	The Cancer Registry 2006
Paper list Gade	178 records (block number) 144-146 persons	Received de-identified with block number	Gade 1997

Annex 4: Table

Raw data linked to information from the Cancer Registry and information on ploidy preparations from the Radium Hospital. Sudbø8 which formed the basis for the article in NEJM 2004 is identical to Raw data which in addition contained prep_no which enabled linkage. Oral cancer is defined by the codes 1400-1499. Bold types shows records in which the date of oral cancer is before the date when the biopsy was taken (date for prep_no). Prep_no is deleted. Missing means that there was no prep_no on the Raw data file.

Raw data	The Commission	Raw data/L29		The Cancer Registry 1996			Sudbø8		Ploidy list
Ptnid record no	unique persons	Prep_no	Year prep	Year cancer	Oral cancer	Year of death	Year leukoplakia	Year cancer	Link to prep_no
1	**P1**		**1981**	**1981**	**Yes**	**1981**	**1982**		**Yes**
2	P2		1981				1990		
3	**P3**		**1981**	**1981**	**Yes**		**1983**	**1986**	
4	**P4**		**1981**	**1981**	**Yes**	**1991**	**1993**	**2000**	**Yes**
5	P5		1981	1981	Yes	1985	1982	1988	Yes
6	**P6**		**1981**	**1981**	**Yes**	**1986**	**1986**		**Yes**
7	P7		1981	1980	No	1993	1992		
8	**P8**		**1981**	**1980**	**Yes**	**1983**	**1993**		**Yes**
9	**P9**		**1982**	**1982**	**Yes**		**1991**	**2001**	
10	P10		1982	1955	No		1991		
11	P11		1982	1973	No	1983	1987	1990	Yes
12	**P12**		**1982**	**1982**	**Yes**	**1983**	**1994**		
13	**P13**		**1982**	**1982**	**Yes**	**1984**	**1992**		
14	P14		1982	1978	No	1983	1994		Yes
15	**P15**		**1982**	**1976**	**Yes**	**1983**	**1990**		
16	**P16**		**1982**	**1982**	**Yes**		**1992**		
17	**P17**		**1990**	**1987**	**Yes**	**1991**	**1993**		
18	**P18**		**1983**	**1975**	**Yes**	**1989**	**1994**		
19	P19		1989	1989	No		1994		
20	**P20**		**1989**	**1989**	**Yes**		**1992**		
21	**P21**		**1983**	**1982**	**Yes**	**1983**	**1993**		**Yes**
22	P22		1983				1992		Yes
23	P23	Missing					1988		
24	**P24**		**1984**	**1984**	**Yes**		**1987**		**Yes**
25	**P25**		**1984**	**1984**	**Yes**	**1984**	**1991**		
26	**P26**		**1984**	**1984**	**Yes**		**1993**		**Yes**
27	**P27**		**1984**	**1984**	**Yes**	**1985**	**1987**		**Yes**
28	**P28**		**1984**	**1984**	**Yes**	**1986**	**1988**		
29	P29		1985	1985	Yes	1986	1990		Yes
30	**P30**		**1985**	**1985**	**Yes**		**1994**		
31	P31		1985				1993		
32	**P32**		**1985**	**1985**	**Yes**	**1993**	**1994**		
33	**P33**		**1985**	**1985**	**Yes**	**1986**	**1994**		
34	P34	Missing					1990		
35	**P35**		**1986**	**1986**	**Yes**		**1993**		**Yes**
36	P36		1986				1982		Yes
37	**P37**		**1986**	**1986**	**Yes**	**1989**	**1987**		**Yes**
38	P38		1986			1987	1991		
39	**P39**		**1986**	**1986**	**Yes**	**1995**	**1993**		
40	P40		1986	1982	No	1992	1985		
41	P41		1986			1987	1994		
42	P42		1986	1986	No	1987	1992		Yes
43	**P43**		**1986**	**1986**	**Yes**	**1988**	**1993**		

Raw data	The Commission	Raw data/L29		The Cancer Registry 1996			Sudbø8		Ploidy list
Ptnid record no	unique persons	Prep_no	Year prep	Year cancer	Oral cancer	Year of death	Year leukoplakia	Year cancer	Link to prep_no
44	**P44**		**1987**	**1987**	**Yes**		**1990**		
45	P45		1987	1988	No	1988	1984	Yes	
46	P46		1987				1986		
47	**P47**		**1987**	**1987**	**Yes**		**1990**	**Yes**	
48	**P48**		**1987**	**1987**	**Yes**	**1992**	**1994**		
49	P17		1987				1983		
50	P49	Missing					1992		
51	**P50**		**1992**	**1992**	**Yes**		**1992**		
51	P41		1986					Yes	
52	P51	Missing					1982		
53	**P52**		**1987**	**1987**	**Yes**	**1988**	**1990**		
54	P44		1987				1994		
55	P45		1987				1987		
56	P46		1987				1984		
57	P47		1987				1983	Yes	
58	P53	Missing					1984		
59	P48		1987				1993		
60	P17		1987				1994		
61	**P54**		**1987**	**1986**	**Yes**		**1994**	**Yes**	
62	**P55**		**1987**	**1986**	**Yes**	**1990**	**1993**	**Yes**	
63	**P56**		**1987**	**1987**	**Yes**		**1990**	**Yes**	
64	P57		1988	1987	No	1992	1989	Yes	
65	P58		1987				1993	Yes	
66	P59		1988				1992		
67	P60		1988	1993	No		1990		
68	**P61**		**1988**	**1988**	**Yes**	**1990**	**1983**	**Yes**	
69	**P62**		**1988**	**1988**	**Yes**		**1985**	**Yes**	
70	P63		1988	1988	No	1990	1990	Yes	
71	P64		1988	1992	No	1992	1988		
72	P65		1988	1989	No		1986	Yes	
73	**P66**		**1988**	**1988**	**Yes**		**1992**	**Yes**	
74	P67		1988	1989	Yes		1982	Yes	
75	P68		1988	1981	No		1987	Yes	
76	**P69**		**1988**	**1988**	**Yes**	**1991**	**1994**		
77	P70	Missing					1990		
78	**P71**		**1989**	**1989**	**Yes**		**1982**	**Yes**	
79	**P72**		**1989**	**1989**	**Yes**	**1993**	**1988**	**Yes**	
80	**P73**		**1989**	**1989**	**Yes**		**1994**		
81	**P74**		**1989**	**1989**	**Yes**		**1987**	**Yes**	
82	P19		1989				1993		
83	P20		1989				1990		
84	**P75**		**1989**	**1979**	**Yes**	**1994**	**1984**		
85	P76		1990				1989	Yes	
86	P77		1990				1987		
87	P78		1990				1988	Yes	
88	P79		1990				1992	Yes	
89	**P80**		**1990**	**1985**	**Yes**	**1991**	**1991**	**Yes**	
90	**P81**		**1990**	**1990**	**Yes**		**1986**		

Raw data	The Commission	Raw data/L29		The Cancer Registry 1996			Sudbø8		Ploidy list
Ptnid record no	unique persons	Prep_no	Year prep	Year cancer	Oral cancer	Year of death	Year leukoplakia	Year cancer	Link to prep_no
91	P82		1990	1990	Yes	1990	1989	Yes	
92	P83		1990				1994		
93	P84		1990	1984	No	1994	1983		
94	P85		1991	1989	No	1992	1984		
95	P86		1991				1993		
96	P87		1991	1991	No	1991	1987		
97	P88	Missing					1992		
98	P89		1991				1991		Yes
99	**P90**		**1991**	**1989**	**Yes**	**1992**	**1983**		**Yes**
100	**P91**		**1991**	**1991**	**Yes**		**1991**		**Yes**
101	P92		1992	1974	No		1986		Yes
102	**P93**		**1992**	**1992**	**Yes**		**1994**		
103	**P94**		**1992**	**1991**	**Yes**		**1993**		**Yes**
104	**P95**		**1992**	**1992**	**Yes**		**1992**		**Yes**
105	P96		1992	1986	No		1988		Yes
106	**P97**		**1992**	**1992**	**Yes**		**1992**	**2000**	**Yes**
107	P98		1992	1992	Yes		1990	2001	Yes
108	**P99**		**1992**	**1992**	**Yes**		**1994**	**1998**	**Yes**
109	P100		1992				1986	1988	Yes
110	**P101**		**1992**	**1982**	**Yes**	**1994**	**1993**		**Yes**
111	**P102**		**1992**	**1992**	**Yes**	**1995**	**1992**	**1996**	
112	P103		1992	1987	No	1995	1994	2000	
113	**P104**		**1992**	**1992**	**Yes**		**1984**	**1987**	
114	P105		1992	1988	No		1993	1997	Yes
115	P106		1992				1990	1993	
116	**P107**		**1992**	**1991**	**Yes**		**1993**	**1997**	
117	**P108**		**1992**	**1992**	**Yes**		**1986**		
118	P109		1992	1980	No	1993	1986		
119	P110		1995				1990		
120	**P111**		**1992**	**1992**	**Yes**		**1986**		**Yes**
121	P112		1993				1984	1988	
122	**P113**		**1993**	**1993**	**Yes**		**1989**	**1992**	**Yes**
123	**P114**		**1993**	**1992**	**Yes**		**1993**	**2000**	**Yes**
124	**P115**		**1993**	**1993**	**Yes**		**1993**	**2001**	
125	**P116**		**1993**	**1993**	**Yes**		**1990**	**1991**	
126	**P117**		**1993**	**1993**	**Yes**		**1994**	**2000**	**Yes**
127	**P118**		**1993**	**1993**	**Yes**		**1989**	**1990**	**Yes**
128	P119		1993				1985		
129	**P120**		**1993**	**1993**	**Yes**		**1982**		
130	P121		1993	1982	No		1991	1992	Yes
131	**P122**		**1993**	**1993**	**Yes**		**1989**	**2000**	**Yes**
132	**P123**		**1993**	**1993**	**Yes**		**1983**	**1984**	
133	**P124**		**1993**	**1993**	**Yes**		**1987**	**1988**	
134	P125	Missing					1994	1995	
135	P126		1994	1968	No		1984	1985	
136	**P127**		**1993**	**1993**	**Yes**		**1990**		**Yes**
137	P128		1993	1984	No	1994	1986		Yes

Raw data	The Commission	Raw data/L29		The Cancer Registry 1996			Sudbø8		Ploidy list
Ptnid record no	unique persons	Prep_no	Year prep	Year cancer	Oral cancer	Year of death	Year leukoplakia	Year cancer	Link to prep_no
138	P129		1994				1983	1988	Yes
139	P130		1994				1991	1993	
140	P131		1994				1994		
141	P132		1994				1986	1988	Yes
142	P133		1994	1981	No		1993	1994	Yes
143	P134		1994				1988	1990	
144	P135		1994				1994		
145	P136		1994				1988	1991	
146	P137		1994	1989	No		1993	1995	
147	P138		1995				1994	1997	Yes
148	P139		1995				1983	1985	Yes
149	P82		1990				1987	1988	Yes
150	P140		1995				1994	1994	

Annex 5: The Dishonesty Committee's Guidelines

Guidelines for the implementation of research projects related to medicine and health[50]

Objective of the guidelines: To provide advice about the design of research plans, documentation and data storage in relation to medical and health research. The objective is to prevent disagreements between project participants and to prevent doubts from being raised about the implementation of a project.

Pre-project checklist:

- Formulate the project's purpose and objectives clearly. Make a project plan (research protocol) which includes what you want to record (effects, variables), how you want to perform the project (method) and the materials and procedures you intend to use.
- Identify any mutuality or conflicts of interest related to the project or among the participants.
- Agree on who is in charge of the project and on the division of labor.
- Agree on who will be writing any publications that are planned. If the situation changes during the project, make a new agreement.
- Clarify questions involving the ownership and user rights to any original data or processed results.
- Any project that includes trials on humans must be submitted to the Regional Research Ethics Committee for Medicine (REK) for approval prior to initiation. Such projects require the informed consent of the subjects in the sample. Draw up your inclusion and exclusion criteria, as well as your criteria for aborting the project. Secure permits for data storage, confidentiality and other relevant conditions, where so required.
- Experiments on animals must be submitted to and approved by the Norwegian Animal Research Authority (NARA) prior to initiation.
- Document equipment that performs measurement-related functions and other measurement instruments, and establish routines for control, calibration and validation.

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[50] Please note that these guidelines are neither entirely up to date nor exhaustive, and that more recent legislation, as for example the Bio Bank Act that came into force in the summer of 2003 is not included.

- Make experiment plans and appurtenant registration forms accessible, straightforward, unambiguous and comprehensible to all those involved in the project. Prepare this material far enough in advance to allow adequate time for training, and possibly for testing and adjustment.

While the project is in progress:
- All parties involved in a project have a mutual obligation to provide information about progress, results, processing, presentation and interpretation.
- Document any deviations from the original investigative plan and experiment procedures. Be sure that any changes in the project or in the potential consequences of any changes are approved by project management.
- Ensure that data on individuals gathered during the course of clinical research projects can be identified and recovered for each individual test subject according to the terms and conditions laid down by the relevant control agency/authorities. The documentation must specify who has collected the data, and when it was collected.
- Ensure that equipment used for measurement is checked and calibrated on a regular basis. The documentation must specify who have checked the equipment, and when.
- Ensure that the materials (e.g. chemicals, preparations. materials) to be used in the project can be identified and documented.

Post-project follow-up:
- Organize original data systematically, safely and so that it is readily recoverable. Pursuant to the national statutory provisions that apply at any given time, licensing terms, contractual terms and conditions, and institution-specific regulations, data, including consent forms, must ordinarily be kept for at least 10 years. The same applies to plans for studies, receipts and descriptions of deviations, if any.
- Once a project is completed, the information may be stored collectively in institutions approved for this purpose. Be sure to sign a final agreement regarding future ownership, storage rights and user rights to data and other material made available as a result of the project. Be especially careful when it comes to person-specific data in order to avoid conflicts related to agreements with the human subjects involved.

If necessary, the owner of the data should be able to establish traceability from published composite data, e.g. tables and figures, to original data.

Medical words and expressions[51]

Adenoma: a benign tumor emanating from and partly structured as a gland

Biopsy: a tissue sample from a living patient for microscope examination

Carcinoma in situ: term for cellular changes that indicate a beginning cancer development without spreading having yet started, a precursor of cancer

Diploid: denoting that a cell contains two sets of chromosomes

DNA aneuploidi: cells with deviating DNA amount, may be found in many malignant tumors

DNA histogram: Graphical presentation of analysis results for DNA amount used in ploidy classification (see separate explanation)

Dysplasia (mild, moderate or severe): a growth abnormality, incomplete of erroneous development of bones, cartilage and/or skin

Erythroplakia: Red patches (plaques) or lesion in the oral mucosa (also called dysplastic leukoplakia); the lesion is related to leukoplakia, but more rare and more serious as it more frequently develops into cancer

Graph theory: Analysis and numerical representation of graphical presentations

Histopathology: The study of pathological changes in tissues

Hydrolysis and Feulgen coloring: Techniques used for the preparation of tissue preparations

Carcinoma: a malignant tumor in epithelial tissues, i.e. in skin, mucosa or glands

Chemoprevention: a term used in particular for intake of various substances to prevent the development of cancer

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[51] Mainly taken from: Nylenna M. Medisinsk ordbok *[Medical Dictionary]*. Oslo: Kunnskapsforlaget, 2005.

Colorectal cancer: Cancer of the large intestine and rectum

Lesion: generic term for circumscribed injuries to the body

Leukoplakia: white patches (plaque) in the oral mucosa that cannot be scraped away, the leukoplakia may develop into cancer

Malignant: cancerous

Malign transformation potential: a lesion with a high malign transformation potential (e.g. erythroplakia) more frequently develops into cancer than a lesion with a lower malign transformation potential (e.g. leukoplakia)

Melanoma: birth mark cancer, pigment cell cancer

NSAID: Non steroidal anti-inflammatory drugs

Odontology: Dental medicine

Oral: Belonging or pertinent to the mouth

Squamous cell carcinoma: Malignant tumor developed from squamous cells in the skin or mucosa

Ploidy classification: the classification of DNA amount as diploid, tetraploid or aneuploid (see separate explanations)

Premalign illnesses: Conditions that may develop into cancer

Re-classification: Repeated classification

Referring letter: a letter from a physician to another physician, hospital, laboratory or the like asking for further care or examination

Tetraploid: Having four sets of chromosomes in the cell nucleus

Tissue block: Paraffin-imbedded tissue used for making histological biopsy specimens.

Statutes and regulations referenced in the report

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Public Administration Act   Lov om behandlingsmåten i forvaltningens saker (LOV-1967-02-10).

Research Ethics Act   Lov om behandling av etikk og redelighet i forskning. (LOV-2006-06-08-71). (Ikke sanksjonert).

Medical Centers Act   Lov om helseforetak m.m. (LOV-2001-06-15-93).

Health Personnel Act   Lov om helsepersonell m.v. (LOV-1999-07-02-64).

Personal Health Data Filing System Act   Lov om helseregistre og behandling av helseopplysninger. (LOV-2001-05-18-4).

Medical Records Regulations   Forskrift om pasientjournal. (FOR-2000-12-21-1385).

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Patients' Rights Act   Lov om pasientrettigheter. (LOV-1999-07-02-63).

Personal Data Act   Lov om behandling av personopplysninger. (LOV-2000-04-14-31).

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Specialist Health Services Act   Lov om spesialisthelsetjenesten m.m. (LOV-1999-07-02-61).

Copyright Act   Lov om opphavsrett til åndsverk m.v. (LOV-1961-05-12-2).

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Summary of the Report of the Commission of Inquiry

1 Appointment
On 18 January 2006, the Rikshospitalet–Radiumhospitalet Medical Center and the University of Oslo (UiO) jointly appointed a special commission to conduct an independent investigation in accordance with detailed terms of reference.

The background for the investigation was that a researcher employed by these institutions, Jon Sudbø, had admitted fabricating the raw data used for a scientific article published in the renowned medical journal The Lancet in October 2005.

2 The investigation
Early in the investigation it became clear that the entire body of Sudbø's scientific work from 1993-2006 (at least 38 publications) would have to be scrutinized, and that the co-authors (60 altogether) would in reality also have to be subject to investigation. All the authors received a letter requesting them to submit a voluntary written statement, which they all did. Moreover, information was gathered from relevant institutions and other relevant partners. Special mention should be made of the findings from the thorough investigations made by the Cancer Registry of Norway. The Commission also met with individuals and representatives of institutions, including Jon Sudbø. Furthermore, the Commission has obtained documents and other information from several other sources. Available data lists, etc., and published research results have been correlated and compared. Accordingly, the Commission was generally able to judge whether, and the extent to which, the underlying data on which the publications are based are genuine. As its main principle, the Commission has found it appropriate to apply a standard of evidence entailing a qualified preponderance of probability as a condition for accepting a particular fact as grounds for the report.

3 Findings
Jon Sudbø began his PhD project in 1993 under the supervision of Albrecht Reith.

The PhD project consists of two separate parts. One part involves theoretical and applied works on tissue architecture in cancerous tumors and normal tissue. The Commission has not found indications of research flaws related to these works.

As reflected in his subsequent research, most of his PhD project involved characterizing the early stages of oral cancer. The research question was whether and, if so, to what extent, different types of classifications of white patches in the oral cavity were indicative of a high risk for developing oral cancer. The doctoral dissertation and related publications give an affirmative response to this question, asserting that a classification based on DNA content can with great accuracy predict the subsequent development of cancer.

First published in the highly respected New England Journal of Medicine in 2001, this sensational finding was based on DNA analyses of 150 patients with leukoplakia (i.e. 'white patches' that may be early stages of oral cancer) in the oral cavity. In 2004, a second article was published in the New England Journal of Medicine, based on further investigations of the same 150 patients. Based on their own investigations and those made by the Cancer Registry of Norway, the Commission's point of departure is that there are serious problems associated with this crucial patient material. For instance, the same patient appears several times. As far as the Commission can determine, the material consists of 141 different patients at the most, since several patients are represented by several tissue samples that collectively add up to 150. Further, the Commission has found that 69 of the 141 patients included in the study should have been excluded because they had been diagnosed with oral cancer before or at the same time as the leukoplakia was diagnosed. For these patients, it was not possible to study the future development of cancer, since they already had cancer. This error alone is so serious that the results and the conclusions are invalid. The Commission has also uncovered several other inconsistencies. For example, the age distribution in the data files is not consistent with the underlying patient material. Further, the Commission has noted that the reported 150 DNA analyses are to some extent repetitions of data from a far smaller number of patients. The reporting on how DNA analyses and the classification of leukoplakia were

conducted (by several observers) is also incorrect and misleading.

Consequently, the Commission has determined that the data underlying parts of the PhD project, as well as several other publications, are not sufficiently consistent with the actual facts the Commission has found it reasonable to take into account. The internal affairs investigation conducted by the Cancer Registry of Norway has arrived at the same conclusion.

The Commission is of the opinion that the errors and defects that have been exposed are too numerous, too great and too obvious to be attributed to random errors, incompetence or the like; and that the raw data therefore appear to have been fabricated, manipulated and adapted to the desired findings.

The consequence of this is that the doctoral dissertation and three related original articles must be retracted. In addition, subsequent publications must be retracted where they are based on the same raw material, as most of them are. On the same grounds, the Commission also questions one other original article. Further, the Commission has questioned an original article published in the Journal of Clinical Oncology 2005, inter alia in the light of circumstances partially acknowledged by Sudbø. The most recent original article published in The Lancet in 2005 has been retracted, since it is, in its entirety, based on fabricated raw data. Jon Sudbø has admitted this.

This means that the bulk of Jon Sudbø's scientific publications are invalid due to the fabrication and manipulation of the underlying data material.

4 Criticism, possible explanations and preventive measures
The exposed fabrication and manipulation of research data justify criticism against Jon Sudbø. The comments that Sudbø has made to the Commission in a meeting and after having read two draft reports with attached documentation, have not given the Commission reason to make any major changes in the preliminary conclusions drawn during the investigation.

In compliance with the terms of reference, the Commission has posed the question of how such – in retrospect – obvious and gross acts could have been perpetuated over such a long period of time in collaboration with so many well-qualified co-authors/scientists and research institutions.

The Commission points out that there will invariably be certain possibilities for a dishonest researcher to dupe and deceive others. Another factor is that Jon Sudbø has operated relatively independently both as a doctoral candidate and later as a researcher. He has always maintained full and sole control of the underlying data. In that connection, the Commission has found reason to criticize his supervisor for a lack of due diligence and academic supervision during Sudbø's fellowship. This case has also revealed what appears to be a systemic failure at the Norwegian Radium Hospital with respect to a lack of supervision, training and control procedures. Another circumstance is that there has been no formal permission or approval whatsoever of the project on the part of external bodies, nor has anyone taken it upon themselves to arrange for or check this. In this context, it has been noted that the institutions that contributed patient material have not required verification of the necessary permits, e.g. dispensation from mandatory confidentiality.

The Commission has not found indications that others, including some of the co-authors, have been involved in the fabrication and manipulation of research data or by other means been party to scientific misconduct. However, in good conscience and based on cost/benefit considerations, the Commission has not perceived its task as being to investigate less serious types of deviations from the norm. The co-authors can generally be divided into two groups: 1) suppliers (subcontractors), and 2) higher level guarantors (senior researchers), who to little or no degree contributed to or had knowledge of the underlying data material. Most communication has taken place through Jon Sudbø. Thus the co-authors have had little opportunity, as well as little reason, to check the underlying data and each other's contributions. Such a division of labour is not uncommon for medical publications that must necessarily be based on cooperation between researchers with rather dissimilar professional backgrounds and tasks, and thus require that they trust each other.

On the other hand, the Commission has pointed out certain factors to which several people should have reacted, be they co-authors, supervisors, superiors, opponents, colleagues or others. Since there have been a number of less serious mistakes on the part of several people that must be viewed in context (collective and cumulative mistakes), the Commission has found reason to view this as systemic failure, where the responsibility rests with the institutions.

In light of this, the Commission has recommended that the institutions take more responsibility for raising awareness and instructing their researchers about the rules that apply, and that they engage in at least a minimum of verification and control, taking appropriate account of academic freedom.

The Commission has not perceived its task as being to expose specific damaging effects. This will probably be a topic for a subsequent investigation by the Norwegian Board of Health. Notwithstanding, the Commission has noted that colleagues, researchers, clinicians and individual patients have probably used Sudbø's research results, and it is therefore reasonable to assume that some of them have been affected. The serious implications of this must have been obvious to Jon Sudbø right from the start.

5 The Commission's Report – an overview
Chapter 2 of the investigative report presents the conditions of the Commission's appointment, the terms of reference and methods of working. The chapter discusses the investigative principle adopted, mode of information retrieval, the principle of contradiction, standards of evidence, the relationship to disclosure, and thresholds for criticism.

In Chapter 3, the Commission has found reason to outline the ethical and legal framework that applies to medical and health research. Here, the Commission provides a general review of the rules of authorship and supervision, etc.

Chapter 4 reviews the facts the Commission has chosen to take into account. The facts are presented in chronological order, beginning with Jon Sudbø's PhD project, which commenced in 1993. There is an explanation of the raw data underlying parts of Jon Sudbø's doctorate and several subsequent publications. The Commission discusses in detail which patient data Sudbø actually had or may have had, comparing it with the data Sudbø and his co-authors stated that they have had in different publications. The Commission then reviewed Sudbø's subsequent scientific publications, which are mainly based on the original raw data from the PhD project.

In Chapter 5, the Commission has attempted to illuminate certain circumstances that may help explain how and why things turned out the way they did.

Chapter 6 offers a brief discussion of the possible consequences of the situation, not least for Norwegian research and patients.

Chapter 7 summarizes the findings and the circumstances worthy of criticism which the Commission has found reason to point out. This criticism refers to individuals and institutions alike.

Finally, the Commission has made certain recommendations in Chapter 8 by way of conclusion.

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Centrosomes in Cellular Regulation

Stephen Doxsey,[1] Dannel McCollum,[2]
and William Theurkauf[1]

[1]Program in Molecular Medicine and [2]Molecular Genetics and Microbiology,
University of Massachusetts Medical School, Worcester, Massachusetts 01605;
email: stephen.doxsey@umassmed.edu; dannel.mccollum@umassmed.edu;
william.theurkauf@umassmed.edu

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spindle pole body, centrosome inactivation, checkpoints,
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network

Abstract

Centrosomes, spindle pole bodies, and related structures in other
organisms are a morphologically diverse group of organelles that
share a common ability to nucleate and organize microtubules and
are thus referred to as microtubule organizing centers or MTOCs.
Features associated with MTOCs include organization of mitotic
spindles, formation of primary cilia, progression through cytoki-
nesis, and self-duplication once per cell cycle. Centrosomes bind
more than 100 regulatory proteins, whose identities suggest roles in
a multitude of cellular functions. In fact, recent work has shown that
MTOCs are required for several regulatory functions including cell
cycle transitions, cellular responses to stress, and organization of sig-
nal transduction pathways. These new liaisons between MTOCs and
cellular regulation are the focus of this review. Elucidation of these
and other previously unappreciated centrosome functions promises
to yield exciting scientific discovery for some time to come.

Contents

INTRODUCTION

Microtubule organizing center (MTOC): structures of diverse morphology that duplicate every cell cycle and nucleate the growth of microtubules

Spindle pole body (SPB): yeast equivalent of the centrosome

Microtubule organizing centers (MTOCs) assume various shapes and sizes and include centrosomes of vertebrate cells and spindle pole bodies (SPB) of yeasts. Despite their diversity of form, all MTOCs share a common function in the nucleation and organization of microtubules. MTOC-organized cytoplasmic microtubules perform a variety of functions, whereas those in mitotic cells play a central role in the organization of mitotic spindles. For more information on MTOC structure, function, and related diseases see Doxsey 2001, Khodjakov & Rieder 2001, Nigg 2002, Sobel 1997. More recent studies demonstrate that MTOCs play new and unexpected roles in several other processes including cell cycle control, cytokinesis, and responses to cellular stress. These burgeoning new areas of centrosome biology are the focus of this review.

CENTROSOMES AND CELL CYCLE CONTROL

Centrosomes (and other MTOCs) are made up of numerous proteins whose amino acid sequence suggests a coiled coil tertiary structure. Increasing evidence indicates that this molecular structure may be well designed for the organization of multiprotein scaffolds that can anchor a diversity of activities ranging from protein complexes involved in microtubule nucleation (Diviani et al. 2000, Diviani & Scott 2001) to multicomponent pathways for cellular regulation (Elliott et al. 1999). By physically linking components of a common pathway, molecular scaffolds can increase the local concentration of components, limit non-specific interactions, and provide spatial control for regulatory pathways by positioning them at specific sites in proximity to downstream targets or upstream modulators. On the basis of the increasing number of regulatory molecules anchored at the centrosome/MTOC (>100), it is likely that this organelle serves as a centralized control center for regulating a diversity of cellular activities. Recent studies have provided some of the first functional links between centrosomes and regulatory networks. In this section, we focus on work that provides the most direct links between centrosomes and cell cycle progression. We discuss the role of the centrosome in cell cycle transitions from G_1 to S-phase, G_2 to M-phase and metaphase to anaphase. The role of centrosomes in progression through cytokinesis is addressed in the next section.

Centrosomes and the G_2 to M Transition

Centrosomal Cdk1 activation during the G_2/M transition. Early work on the G_2/M transition showed that centrosomes could induce progression into mitosis following injection into G_2-arrested starfish oocytes (Picard et al. 1987). Centrosomes were also able to activate maturation promoting factor [MPF, now known as cyclin-dependent kinase1-cyclinB complexes (Cdk1-cycB)] and induce premature mitotic entry in *Xenopus* eggs (Perez-Mongiovi et al. 2000). Other studies showed that mitotic kinases and cyclins were present at centrosomes. More recent work indicates that centrosomes are involved in the G_2 to M transition in mammalian cells. Mitosis is initiated in part by activation of Cdk1-cycB. CycB1 is present throughout the cytoplasm prior to prophase. However, active Cdk1-cycB1 is first detected at the centrosome during prophase and prior to the Cdk1-cycB1-dependent phosphorylation of histone H3 in the nucleus (Jackman et al. 2003). This observation suggested that centrosomes might function as sites of integration and activation of proteins that trigger mitosis.

A clever centrosome targeting strategy was recently used to provide evidence that mitotic entry requires centrosome localization of Cdk1 and its modulators (Kramer et al. 2004). The authors demonstrated that the checkpoint kinase 1 (Chk1) associated with centrosomes in nonmitotic cells and inhibited Cdk1 activity. Inhibition of Chk1 induced premature activation of Cdk1 at centrosomes and premature mitotic entry. When the centrosome localization sequence (CLS) of the centrosome protein AKAP450 was fused to Chk1, the kinase was immobilized at the centrosome where it was unable to phosphorylate nuclear substrates. Chk1 lacking the CLS did not localize to centrosomes and phosphorylated substrates normally. Centrosome immobilization of a kinase-inactive form of Chk1 induced premature Cdk1 activation

and premature mitotic entry, whereas centrosome immobilization of wild-type Chk1 prevented Cdk1 activation at centrosomes and induced mitotic failure, polyploidy, and multiple centrosomes.

The Chk1-mediated inhibition of Cdk1 activity was not direct but linked to inhibition of the Cdk1 activating phosphatase, Cdc25B (Kramer et al. 2004). Cdc25-mediated activation of Cdk1 seemed to occur through centrosome-localized Chk1 (Forrest et al. 1999, Kramer et al. 2004). In addition, aurora-A kinase was required for recruitment of Cdk1-cycB to centrosomes and thus, for activation of the kinase (Hirota et al. 2003). Centrosome-associated Polo kinase (Polo) is also involved in mitotic entry, although it does not appear to be directly linked to Cdk1 activation. Localization of Polo to centrosomes during G2/M depends upon the polo box (Jang et al. 2002, Lee et al. 1998, Reynolds & Ohkura 2003), which prevents mitotic entry and arrests cells with 4N DNA content when overexpressed. In contrast, polo box mutants do not localize to centrosomes or inhibit mitotic progression (Jang et al. 2002).

Taken together, these results suggest that there is a cell cycle regulatory module at the centrosome that integrates positive and negative pathways to control mitotic entry. However, removal of centrosomes/centrioles does not prevent entry into mitosis (Hinchcliffe et al. 2001, Khodjakov & Rieder 2001), suggesting that centrosomal regulation of Cdk1 activation by Chk1 may not be required for mitotic entry. On the other hand, the pericentriolar material (PCM) and other material remaining in the acentriolar MTOC after extraction of the centrioles may serve this function (Hinchcliffe et al. 2001, Khodjakov & Rieder 2001). Thus it seems that centrosome-associated regulatory pathways may be dominant over centrosome-independent pathways for mitotic entry (Kramer et al. 2004).

Centrosomal γ tubulin and the G_2/M transition. Centrosomal microtubule nucleation is mediated in part by γ tubulin ring complexes

Cdk: cyclin-dependent kinase

Cyc: cyclin

Centrioles: microtubule-based structures comprised of α/β tubulin and other proteins surrounded by pericentriolar material

Pericentriolar material (PCM): fibrillar material surrounding centrioles in the centrosome that

(γTuRCs). Pericentrin is a coiled coil scaffolding protein that anchors γTuRCs at centrosomes (Zimmerman et al. 2004). Uncoupling of the pericentrin-γTuRC interaction by peptides encoding pericentrin's γTuRC-binding domain, or by siRNA (small interfering RNA)–mediated pericentrin depletion, induced arrest at G_2/M followed by apoptosis. Some cell lines appeared insensitive to the G_2/M arrest, continued to cycle, and revealed a reduction in centrosomal γtubulin and astral microtubules in mitosis. It will be interesting to determine if centrosome-associated regulatory molecules involved in cell cycle progression are mislocalized from centrosomes under these conditions (see above).

The Centrosome/Spindle Pole in the Metaphase to Anaphase Transition

Although the kinetochore is best known for sensing and regulating the metaphase to anaphase transition (Maiato et al. 2004), the centrosome/spindle pole also appears to play a role in this process. The was first suggested by data showing that destruction of GFP-tagged and endogenous cycB at the M-A transition in *Drosophila* was initiated at the spindle pole, which then proceeded up the spindle (Huang & Raff 1999). When centrosomes were lost from spindles in the *Drosophila* mutant *cfo*, cycB was lost from centrosomes but not spindles, and cells arrested in anaphase (Wakefield et al. 2000). Recent work in cellularized *Drosophila* embryos showed that Fzy/Cdc20 was responsible for the spindle-associated wave of cycB destruction, whereas Fzr/Cdh1 was required for destruction of cytoplasmic cycB (Raff et al. 2002).

More recent studies have implicated the centrosome proteins γtubulin and the pericentrin B homologue, Pcp1p, in regulation of the M-A transition. In *Aspergillus nidulans*, a cold-sensitive γtubulin allele did not inhibit spindle formation at the restrictive temperature but delayed the M-A transition and induced cytokinesis failure (Prigozhina et al. 2004). Likewise, a mutation in *Schizosaccha-*

romyces pombe, Pcp1p, inhibited the M-A transition without disrupting bipolar spindle assembly (Rajagopalan et al. 2004). This it appears that centrosomal pericentrin homologs and γtubulin complexes may be involved in regulation of multiple cell cycle transitions (G_2/M and M-A).

The Centrosome in the G_1 to S-phase Transition

Removal of centrioles and other core centrosome components induces G_1 arrest. Recent data indicate that centrosomes can affect progression through the cell cycle (**Figure 1**). Studies designed to remove centrosomes/centrioles from cells by microsurgical cutting (Hinchcliffe et al. 2001) or laser ablation (Khodjakov & Rieder 2001) have provided direct evidence for centrosomes in cell cycle progression. Under both conditions, the centriole pair and associated proteins were removed, yet it is important to note that these cells formed acentriolar MTOCs containing proteins of the PCM and perhaps other centrosome structures (Khodjakov & Rieder 2001, Khodjakov et al. 2002). Similar acentriolar MTOCs are functional in higher plants and some animal meiotic systems (Shimamura et al. 2004, Theurkauf & Hawley 1992). In mammlian cells with acentriolar MTOCs, early mitotic events occurred normally but many cells exhibited cytokinesis defects or failure. All cells with acentriolar MTOCs generated by microsurgery failed to initiate DNA replication (BrdU-negative) regardless of whether they completed cytokinesis. Moreover, ablation of one of two centrosomes in prometaphase cells produced a centrosome-containing daughter that continued to cycle and a daughter cell with an acentriolar MTOC that arrested in G_1 (BrdU-negative). Both strategies used to remove core centrosome structures and components showed that acentriolar MTOCs experience problems during cytokinesis and subsequently undergo G_1 arrest. However, extra centrosomes created by cell fusions or inhibition of
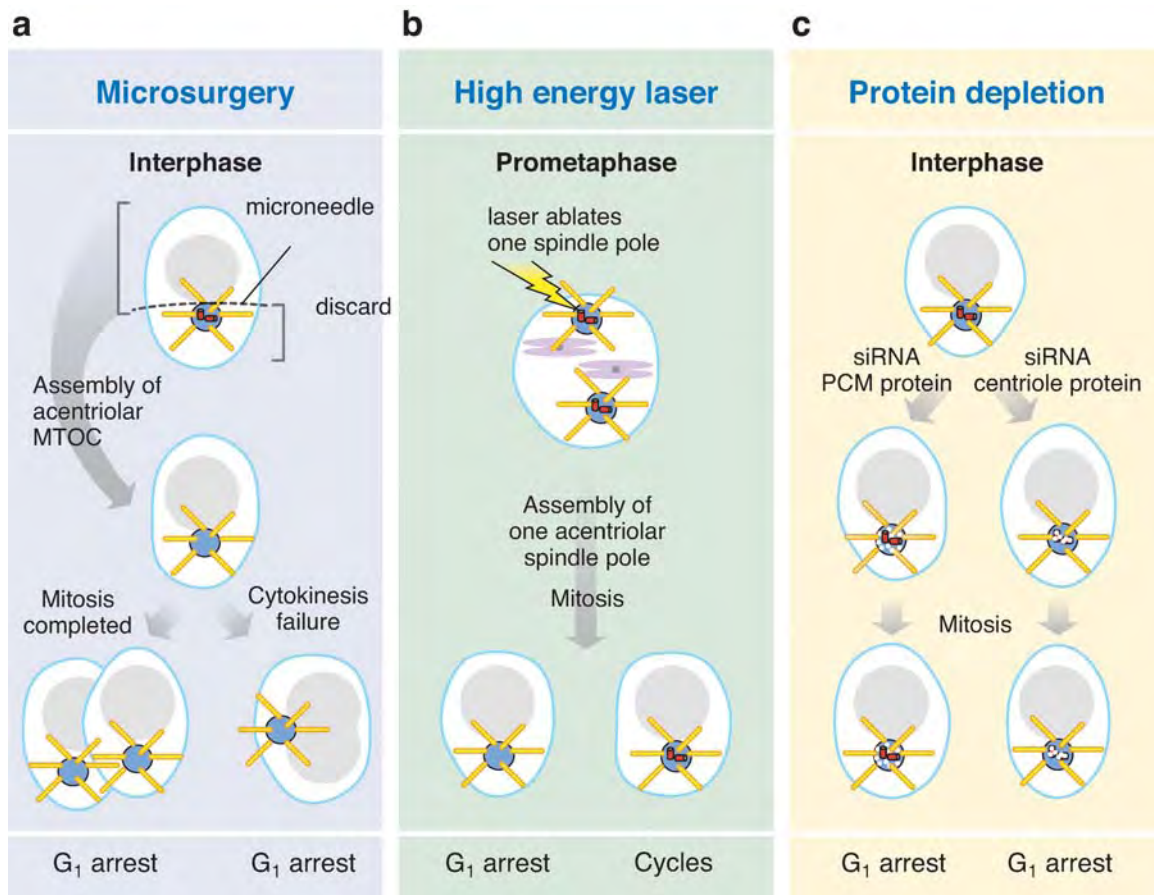
Figure 1

Centrosomes are required for G_1 to S-phase progression. Removal of centrosome (*blue/red*) by (*a*) microsurgery or (*b*) laser ablation produces cells with loosely focused microtubule arrays (*bottom row*, *yellow*) organized by an MTOC-containing PCM proteins (*blue*) but lacking centrioles (*red*). Acentriolar MTOC-containing cells undergo G_1 arrest. (*b*) Cells with intact centrosomes cycle (*right*). (*c*) Depletion of centrosome proteins of the centrioles (*red turned white*), PCM (*blue turned white*), or other structures by small interfering RNAs (siRNA) results in G_1 arrest (*bottom row*). Nucleus, gray (adapted from *Trends in Cell Biology*, in press).

cytokinesis does not inhibit cell cycle progression (Uetake & Sluder 2004, Wong & Stearns 2003). Similarly, centrosome-associated microtubules do not appear to play an essential role in cell cycle progression. Addition of the microtubule depolymerizing drug nocodazole to normal cycling diploid cells (2N) did not induce arrest in G_1 of cells with diploid genomes (2N), even after they were first synchronized in G_1/G_0 by serum starvation. Instead, nocodazole-treated cells continued to cycle, delayed in mitosis, experienced mitotic failure, and then arrested as tetraploid cells (4N) in a G_1-like state (Lanni & Jacks 1998, Trielli et al. 1996). Because G_1 arrest was observed after mitotic failure in tetraploid cells with multiple centrosomes, it will be important to confirm this result under physiological conditions using normal cycling diploid cells.

Changes in centrosome protein levels or localization induce G$_1$ arrest. Recent studies have identified centrosome proteins that function in cytokinesis and cell cycle progression (**Figure 1**). Centriolin is a centrosome protein that shares homology with yeast proteins involved in cytokinesis and mitotic exit (Nud1p and Cdc11p) (Gromley et al. 2003, Guertin et al. 2002). Overexpression of the Nud1p/Cdc11p homology domain or centriolin depletion induced cytokinesis defects followed by cell cycle arrest in G$_1$ as shown by flow cytometry and BrdU staining. A similar cell cycle arrest in G$_1$ was observed when the centrosome protein AKAP450 and PKA were mislocalized from centrosomes (Gillingham & Munro 2000, Keryer et al. 2003). The data thus far show that removal of entire centrioles or changes in individual centrosome proteins induce cytokinesis defects that appear to lead to G$_1$ arrest.

SiRNA-mediated depletion of several centrosome proteins induces G$_1$ arrest. As discussed above, centrosomes play key roles in spindle function and cytokinesis. This suggests that the G$_1$ arrest observed after perturbation of centrosomes or centrosome proteins may result from mitotic dysfunction. Recent data provided evidence for a mitosis-independent cell cycle arrest. Antibodies to the centrosome protein PCM-1 prevented entry into S-phase when microinjected into early interphase mouse zygotes (Balczon et al. 2002). In another study, RNA interference was used to individually deplete more than 20 centrosome proteins that localized to several independent centrosome sites (e.g., PCM, centriole) (**Figure 1**). Depletion of nearly all these proteins induced G$_1$ arrest as shown by accumulation in the 2N peak by flow cytometry, lack of BrdU incorporation, and reduction in the activity of cyclin-dependent kinase2-cyclinA/E complexes (Cdk2-cycA/E) (Mikule et al. 2003). G$_1$ arrest could be rescued by returning targeted centrosome proteins to normal levels. No common functional change was observed for proteins that induced cell cycle

arrest. These results suggested that cell cycle arrest could be induced through discrete alterations in centrosome composition.

G$_1$ arrest is induced in postmitotic cells. The role of mitotic dysfunction in G$_1$ arrest was examined in more detail using postmitotic cells (early G$_1$). Cells that had recently completed cytokinesis were microinjected with a plasmid encoding the centrosome-targeting region of pericentrin/AKAP450 that mislocalizes both proteins from centrosomes (Gillingham & Munro 2000). This reduced the centrosome-bound fraction of endogenous pericentrin and prevented cells from entering S-phase and incorporating BrdU (K. Mikule & S. Doxsey, unpublished observations). Another recent study showed that ablation of centrosomes in postmitotic cells inhibited progression through the cell cycle (14/16 cells; A. Khodjakov, personal communication). Taken together, results from multiple approaches show that G$_1$ arrest can be induced from within G$_1$.

Possible mechanisms of centrosome-associated G$_1$ arrest. How centrosome loss or alteration leads to G$_1$ arrest is currently unclear. Regulation of centrosome duplication and entry into S-phase are similar in that both require Cdk2-cycE/A complexes (Lacey et al. 1999). As described above, ectopic expression of cycE accelerated entry into S-phase whereas expression of the cycE centrosome localization domain (CLD) disrupted centrosome binding of endogenous cycE (and cycA) and prevented entry into S-phase (Matsumoto & Maller 2004). We currently do not know if centrosome targeting of cycE is lost during centrosome removal or centrosome protein depletion, and if this contributes to the centrosome-induced G$_1$ arrest. In this regard, the centrosome could control S-phase entry and therefore the nuclear replication cycle through mechanisms such as cycE binding that are independent of the cell cycle because cell cycle arrest by cycE CLD does not require Cdk2 binding.

A centrosome-induced checkpoint? Studies on centrosome protein depletion suggest that G_1 arrest involves activation of a cell cycle checkpoint. For example, G_1 arrest was suppressed in human tumor cells with abrogated p53 function and in cells acutely depleted of p53 following centrosome protein depletion (K. Mikule & S. Doxsey, unpublished observation). In addition, the p38 stress-activated signal transduction pathway was shown to be involved in the G_1 arrest. These observations suggest that the inability of some cells to arrest in the absence of centrioles may be related to loss or abrogation of p53 or p38 or related regulatory molecules (Bobinnec et al. 1998, Piel et al. 2001). It is also possible that checkpoint signaling may occur at the centrosome given the localization of both p53 and p38 to this site (Ciciarello et al. 2001, Liu et al. 2004; K. Mikule & S. Doxsey, unpublished observations). Tumor cells with abrogated p53 or p38 function may avoid checkpoint activation, continue to cycle and propagate centrosome defects, mitotic dysfunction, and genetic instability.

ROLE OF CENTROSOMES AND SPINDLE POLE BODIES IN CYTOKINESIS AND MITOTIC EXIT

Recent studies have implicated SPBs and centrosomes in numerous aspects of cell cycle progression including mitotic exit and cytokinesis. Studies in both the fission yeast *S. pombe* and the budding yeast *Saccharomyces cerevisiae* have delineated two conserved signaling pathways termed the septation initiation network (SIN) and the mitotic exit network (MEN), respectively, which localize to the SPBs and regulate cytokinesis and mitotic exit. Both of these pathways have been covered extensively in recent reviews (Seshan & Amon 2004, Simanis 2003) and are not be reviewed in detail here. Instead, we focus on more recent studies, novel functions for these pathways, and potential conserved functions in mammalian cells.

The SIN Pathway in *S. pombe*

As mentioned above, the SIN pathway is essential for cytokinesis in fission yeast (**Figure 2**). The SIN is a SPB localized-GTPase-regulated protein kinase cascade (for a list of SIN homologs in *S. pombe*, *S. cerevisiae*, and mammalian cells, see **Table 1**). SIN mutants proceed normally through the cell cycle and mitosis and can form cytokinetic actomyosin contractile rings, a key structure required for cytokinesis analogous to the cleavage furrow in animal cells. However, SIN mutants fail to initiate constriction of the ring and the rings fall apart causing the cells to fail cytokinesis and become multinucleate (Balasubramanian et al. 1998, Fankhauser et al. 1995). Inappropriate activation of the SIN can also drive exit from mitosis, suggesting that the SIN may play a nonessential role in exit from mitosis (Fankhauser et al. 1993, Guertin et al. 2002).

Asymmetry of SIN signaling. One curious feature of the SIN is the asymmetric pattern of loca lization of some components to the SPBs (**Figure 2**). It has been unclear both how the asymmetry of the SIN pathway is generated as well as why SIN signaling is asymmetric. Although there is still little known about the functional significance of asymmetry for SIN signaling, a recent study revealed the basis of the asymmetry of the SIN pathway (Grallert et al. 2004). This study found that the asymmetry of the SIN reflected underlying asymmetry of the spindle poles. After SPB duplication, each cell has an old SPB that was inherited from the previous cell cycle and the new SPB that forms upon SPB duplication. It was discovered that in anaphase, when the asymmetry of the SIN arises, the SIN inhibitors Cdc16p-Byr4p localize to the old SPB, whereas the SIN activator Cdc7p and presumably Sid1p-Cdc14p localize to the new SPB. It is not known why SIN signaling is asymmetric, but it may have to do with down regulating the SIN because most of the known mutations that activate SIN signaling

Septation initiation network (SIN): signaling network in *S. pombe*, analogous to MEN in *S. cerevisiae*, required for cytokinesis

Mitotic exit network (MEN): signaling network required for mitotic exit in the budding yeast *S. cerevisiae*
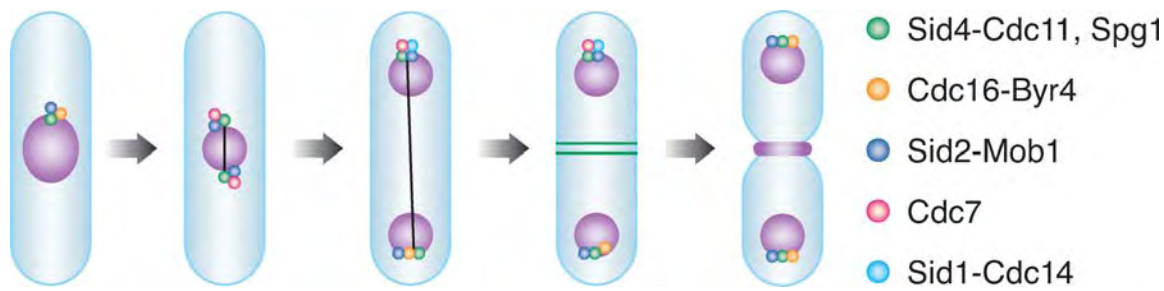
Figure 2

Cell cycle–dependent localization of SIN components. All SIN components localize to the spindle pole body (SPB). Sid4p and Cdc11p form a complex at the SPB that functions as a scaffold required for localization all known SIN components to the SPB (Chang & Gould 2000; Guertin et al. 2000; Hou et al. 2000; Krapp et al. 2001, 2004; C. Li et al. 2000; Morrell et al. 2004; Sparks et al. 1999). Sid4p-Cdc11p and Spg1 (*green*), and to some extent Sid2p-Mob1p (*blue*) localize to the SPB throughout the cell cycle. SIN signaling is negatively regulated by Cdc16p and Byr4p (*orange*), which function as part of a two-component GTPase activating protein (GAP) for Spg1p (Furge et al. 1998). A guanine-nucleotide exchange factor (GEF) for Spg1p has not been identified. Both the activity and localization of SIN proteins is regulated through the cell cycle. In interphase the Cdc16p-Byr4p GAP complex localizes to the SPB (Cerutti & Simanis 1999, C. Li et al. 2000) and, consistent with this, Spg1p is at the pole but in the inactive GDP-bound state (Sohrmann et al. 1998). As the mitotic spindle forms during metaphase, Spg1p becomes activated at both SPBs (GTP-bound form), and Cdc16p-Byr4p leave both SPBs (Cerutti & Simanis 1999, C. Li et al. 2000). Cdc7p (*red*) is recruited to the SPB by the GTP-bound form of Spg1p to which it binds directly (Sohrmann et al. 1998). However, the SIN does not become activated at this time. SIN activation in metaphase is restrained by Cdk activity. During anaphase B, after Cdk inactivation, Cdc16p-Byr4p returns to one SPB (Cerutti & Simanis 1999, C. Li et al. 2000). Spg1p is inactivated, and Cdc7p becomes delocalized at that SPB. Also at this time Sid1p-Cdc14p (*aqua*) localizes to the single Cdc7p containing SPB in anaphase (Guertin et al. 2000). Once Sid1p-Cdc14p localizes to the SPB, it and possibly Cdc7p are then presumed to activate Sid2p-Mob1p and cause them to translocate to the actomyosin ring to trigger ring constriction and septation (Guertin et al. 2000, Sparks et al. 1999). Once septum formation is complete, the SIN becomes inactive and returns to its interphase configuration.

result in localization of SIN activators to both SPBs.

The SIN, CDK regulation, and the cytokinesis checkpoint. The SIN is kept inactive in early mitosis by Cdk activity (Chang et al. 2001, Guertin et al. 2000). Cdk inhibition of the SIN may be from direct phosphorylation of SIN components by Cdk1, since Cdk1p-Cdc13p binds directly to the SIN scaffold protein Cdc11p, positioning it to phosphorylate SIN proteins (Morrell et al. 2004). The targets of Cdk phosphorylation in the SIN are not known. Because, in *S. pombe* and most other organisms, Cdk1p inactivation occurs coincident with chromosome segregation, coupling initiation of cytokinesis to Cdk1p inactivation ensures that cell division does not initiate

before chromosomes have been segregated. However, this mechanism renders cytokinesis sensitive to Cdk activity, which begins to rise shortly after completion of cytokinesis, as the next cell cycle initiates. If cytokinesis is delayed, the rising Cdk1p activity could inhibit the SIN and cytokinesis unless the cell has a way to inhibit Cdk activity until cytokinesis is complete. Clp1p/Flp1p, the *S. pombe* homolog of the budding yeast Cdc14 phosphatase homolog Clp1p/Flp1p (hereinafter referred to as Clp1p) plays a crucial role in maintaining SIN activity if cytokinesis is delayed (Mishra et al. 2004, Trautmann et al. 2001). Because Cdc14-family phosphatases dephosphorylate sites phosphorylated by Cdks (Esteban et al. 2004, Kaiser et al. 2002, L. Li et al. 2000, Visintin et al. 1998, Wolfe & Gould 2004),

Clp1p presumably maintains SIN signaling when cytokinesis is delayed by antagonizing the inhibitory effects of Cdk phosphorylation on the SIN. Clp1p also inhibits Cdk activity by dephosphorylating and destabilizing Cdc25p (Esteban et al. 2004, Wolfe & Gould 2004). Through this mechanism Clp1p antagonizes Cdk1 activity by promoting inhibitory tyrosine phosphorylation on Cdk1.

Clp1p localizes to the nucleolus in interphase where it is thought to be sequestered and inactive (Cueille et al. 2001, Trautmann et al. 2001). Clp1p is released from the nucleolus in mitosis and the SIN acts to keep Clp1p out of the nucleolus until cytokinesis is complete. Thus Clp1p and the SIN seem to function together, each acting to maintain the others activity until cytokinesis is complete. This suggests that the SIN inhibits cell cycle progression through Clp1p. However recent evidence showed that hyperactivation of the SIN can block cell cycle progression independently of Clp1p, suggesting that the SIN can antagonize Cdk activity through another mechanism (Mishra et al. 2004). This activity becomes crucial when cytokinesis is delayed. When cytokinesis is delayed in wild-type cells, the SIN remains active, Clp1p stays out of the nucleolus, the cytokinetic apparatus is maintained, and the cells arrest further nuclear division until cytokinesis is complete. This ensures that the cell does not become multinucleate and polyploid if cytokinesis is delayed. Complete inhibition of cytokinesis results in a prolonged delay in nuclear division (Cueille et al. 2001, Trautmann et al. 2001). In contrast, cells with weakened SIN signaling, or deletion of *clp1*, are not to be able to maintain the cytokinetic apparatus in response to delays in cytokinesis, and the cytoskeleton returns to the interphase configuration. These cells then proceed with further rounds of nuclear division resulting in multinucleate, polyploid cells. This is reminiscent of the delay or block in cell cycle progression observed in mammalian cells after failure to complete mitosis or cytokinesis (for review, see Stukenberg 2004).

Table 1   SIN and MEN proteins and potential mammalian homologs

S. pombe	*S. cerevisiae*	Mammals	Protein function
plo1	CDC5	Polo	Kinase
sid4	Unknown	?	SPB scaffold
cdc11	NUD1	centriolin	SPB scaffold
spg1	TEM1	?	GTPase
cdc7	CDC15	Mst2?	Kinase
sid1	Unknown	Mst2?	Kinase
cdc14	Unknown	?	Sid1 binding
sid2	DBF2	Warts1/Lats1, Lats2	Kinase
mob1	MOB1	Mob1	Sid2/Dbf2 binding
clp1	CDC14	Cdc14A/B	Phosphatase
cdc16	BUB2	?	Part of GAP
byr4	BFA1/BYR4	?	Part of GAP

The SIN consists of two scaffolding proteins (Sid4p, Cdc11p) (Krapp et al. 2004, Morrell et al. 2004), four protein kinases (Plo1p, Cdc7p, Sid1p, and Sid2p) (Fankhauser & Simanis 1994, Guertin et al. 2000, Ohkura et al. 1995, Sparks et al. 1999) and one small GTPase, Spg1p (Schmidt et al. 1997). Additionally, the Cdc14p (Fankhauser & Simanis 1993) and Mob1p (Hou et al. 2000, Salimova et al. 2000) proteins function as subunits of the Sid1p and Sid2p kinases, respectively. The MEN consists of a scaffolding protein (Nud1p) (Gruneberg et al. 2000), four protein kinases (Cdc5p, Cdc15p, Dbf2p, and Dbf20p), a GTPase (Tem1p), an exchange factor (Lte1p), a protein phosphatase (Cdc14p), and a Dbf2p-binding protein (Mob1p) (Johnston et al. 1990; Kitada et al. 1993; Luca & Winey 1998; Schweitzer & Philippsen 1991; Shirayama et al. 1994a,b; Toyn et al. 1991; Wan et al. 1992). Potential homologs of several SIN/MEN components have been identified, including centriolin (Gromley et al. 2003), Polo kinase (Golsteyn et al. 1994), Cdc14A/B (Li et al. 1997), Mst2 kinase (Hay & Guo 2003), Mob1 (Luca & Winey 1998), Warts/Lats1 (Nishiyama et al. 1999, Tao et al. 1999), and Lats2 (Hori et al. 2000, Yabuta et al. 2000) kinases.

See below for speculation about whether homologs of the SIN/MEN proteins y function in the mitosis to interphase transition in mammalian cells to deal with mitotic or cytokinetic failures.

The MEN Pathway in *S. cerevisiae*

In *S. cerevisiae* the pathway analogous to the SIN is termed the MEN. Like the SIN, the MEN is a GTPase-regulated protein kinase cascade, whose components localize to both the SPB and the bud neck (Seshan & Amon 2004, Simanis 2003). Mutants in the MEN pathway arrest at the end of anaphase with elongated spindles and high Cdk activity. The MEN is required to antagonize Cdk

activity and bring about mitotic exit. Similar to the SIN, the MEN also plays a role, albeit nonessential, in cytokinesis. The MEN functions in anaphase to inhibit Cdk activity and cause mitotic exit by promoting release of the Cdc14p phosphatase from the nucleolus by an unknown mechanism (Shou et al. 1999, Visintin et al. 1999). Cdc14p then dephosphorylates a number of Cdk substrates including Cdh1p/Hct1p and the Cdk inhibitor Sic1p (Visintin et al. 1998). Dephosphorylation by Cdc14p stabilizes Sic1p and also causes activation of Cdh1p/Hct1p, which promotes cyclin B proteolysis. Together, these events result in Cdk inactivation and exit from mitosis.

Regulation of MEN activity. An elegant model has been proposed for MEN activation in anaphase, where elongation of the spindle into the bud would bring the SPB localized Tem1p GTPase into contact with its bud localized activator Tem1p (Bardin et al. 2000, Pereira et al. 2000) (**Figure 3**). This model would allow for temporal and spatial coupling of mitotic exit and chromosome segregation, such that exit from mitosis occurs only after chromosomes have segregated to each daughter cell. Some recent evidence suggests that this model may not be quite so straightforward. Lte1p has not been shown to directly activate Tem1p in vitro, and further experiments suggest that its exchange activity may not be
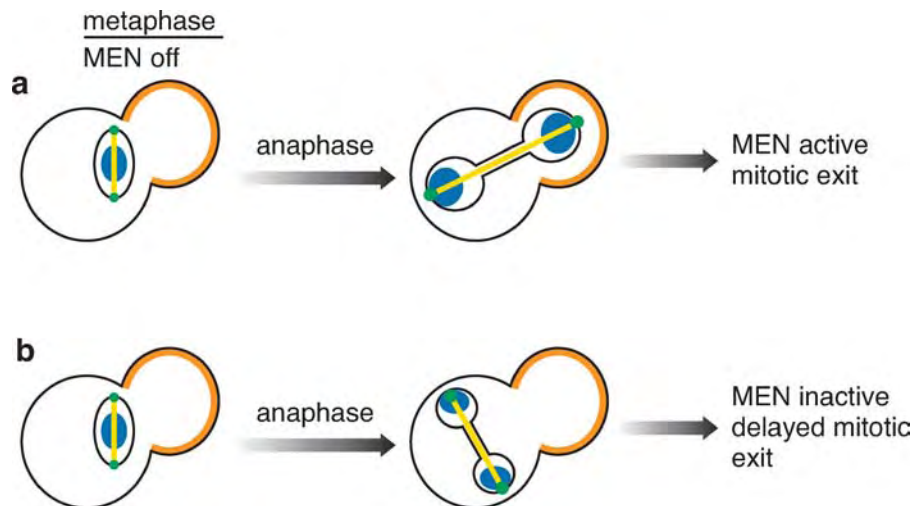
Figure 3

Regulation of MEN activity. (*a*) Several mechanisms function to keep the MEN inactive in metaphase. Premature activation of the MEN in metaphase is prevented by the Bub2p-Bfa1p, which serves as a GAP for the Tem1p GTPase (Alexandru et al. 1999, Fesquet et al. 1999, Fraschini et al. 1999, Li 1999). Additionally, Tem1p is spatially separated from its proposed GTP exchange factor Lte1p, which localizes to the bud cortex (*orange*). Cdk phosphorylation of Cdc15p is also thought to antagonize MEN signaling in metaphase (Jaspersen & Morgan 2000, Menssen et al. 2001). Similarly, activation of the MEN in anaphase is brought about by multiple mechanisms. Activation of the MEN depends on the passage of one of the spindle poles through the bud neck during anaphase spindle elongation (Molk et al. 2004, Yeh et al. 1995). Once this has occurred, it brings that pole in proximity to the putative exchange factor Lte1p, which localizes specifically to the bud cortex (Bardin et al. 2000, Pereira et al. 2000), presumably allowing activation of the Tem1p GTPase. Although some recent results raise questions about certain aspects of this model (see text), it provides a nice explanation for how spindle orientation in anaphase is coupled to mitotic exit. (*b*) Cells with defects in spindle orientation often undergo anaphase chromosome separation in the mother cell. These cells delay MEN activation and exit from mitosis until correct spindle orientation is achieved. Correct spindle orientation results in exit from mitosis, presumably in part by bringing SPB localized Tem1p into contact with bud localized Tem1p (*orange*).

required for its role in mitotic exit (Yoshida et al. 2003), raising questions about whether Lte1p is the GEF for Tem1p. Also, Lte1p is essential only for mitotic exit at low temperatures, indicating that Tem1p can become activated in the absence of Lte1p. Thus other mechanisms may contribute to activation of the MEN, including reduced Cdk inhibition of the MEN that occurs when Cdk activity decreases in anaphase through partial proteolysis of B-type cyclins and early anaphase release of the Cdc14p phosphatase triggered by the FEAR network (see D'Amours & Amon 2004 for review). It has also been proposed that the loss of astral microtubules from the bud neck that occurs when the spindle elongates through the bud neck functions independently from Lte1p to promote mitotic exit (Castillon et al. 2003). In addition, Cdc5p, the *S. cerevisiae* polo homolog, acts upstream to antagonize Bub2p/Byr4p by phosphorylating Byr4p directly facilitating activation of the MEN (Hu et al. 2001). Together, these studies suggest that regulation of the MEN is complex and controlled by numerous inputs.

Asymmetry at SPBs and MEN regulation. Although not all reports are in agreement, the MEN components Bub2p and Tem1p seem to preferentially localize to the SPB that enters the bud (Bardin et al. 2000, Pereira et al. 2000). Recent analysis (Molk et al. 2004) of localization of GFP fusions in live cells shows that Bub2p, Tem1p, and Cdc15p show asymmetric localization to the SPB in the bud. As the old SPB enters the bud, Tem1p localization increases at that site. This coincides with Cdc15 localization to the same SPB, which is presumably recruited by Tem1p. Surprisingly, localization of the Tem1p inhibitor Bub2p also increases at the old SPB as it passes into the bud. The reason for the asymmetry is not clear. It is presumed that asymmetry is important for MEN signaling. In dynein mutants, where anaphase takes place in the bud, Tem1p localizes in a symmetric manner to both SPBs, and the MEN remains inactive. It is curious that both MEN and the SIN proteins local-

ize asymmetrically on the SPBs, but the active SIN components are on the new SPB, whereas the MEN components are on the old SPB. However, as with the SIN, proof of the importance of asymmetry in MEN signaling is lacking.

MEN and cytokinesis. Recent evidence suggests that the MEN, similar to the SIN, also plays a more direct role in cytokinesis, although this function does not appear essential. Cells in which the requirement for the MEN in mitotic exit has been relieved are viable, but they still have defects in cytokinesis (Lippincott et al. 2001, Luca et al. 2001, Menssen et al. 2001). Moreover, certain alleles of *cdc15* and *mob1* have been identified that are specifically defective for cytokinesis (Jimenez et al. 1998, Luca et al. 2001, Menssen et al. 2001). The intracellular localization of some MEN components are also consistent with a role in cytokinesis as Cdc15p (Xu et al. 2000), Cdc5p (Cheng et al. 1998, Song et al. 2000), and Dbf2p-Mob1p (Frenz et al. 2000, Luca et al. 2001, Yoshida & Toh-e 2001) have been observed to localize at the bud neck. The MEN is required to promote splitting of the septin ring and constriction of the actomyosin ring in telophase (Cid et al. 2001, Lippincott et al. 2001); however, as with the SIN, it is not yet clear at a molecular level how the MEN triggers cytokinesis.

There is also evidence that MEN function in cytokinesis is inhibited by Cdk activity, similar to the SIN in *S. pombe*. For instance, the MEN promotes release of Cdc14p from the nucleolus; however. Cdc14p also seems to promote the cytokinesis function of the MEN. In early mitosis, Cdc15p is phosphorylated by Cdk1, and in late mitosis, as Cdk activity drops, Cdc15p becomes dephosphorylated by Cdc14p (Menssen et al. 2001, Xu et al. 2000). Dephosphorylation of Cdc15p seems to be important for its ability to promote cytokinesis but not mitotic exit (Menssen et al. 2001). Cdc14p may also be important for regulating Dbf2p-Mob1p localization, because Dbf2p becomes activated in *cdc14* mutants

(Lee et al. 2001, Mah et al. 2001), but does not localize to the bud neck (Frenz et al. 2000, Yoshida & Toh-e 2001). This is reminiscent of observations in *S. pombe* showing that the SIN is inhibited by Cdk activity to ensure that cytokinesis does not occur until exit from mitosis is complete.

Centrosomes and Cytokinesis in Animal Cells

Although the centrosome has not been implicated in exit from mitosis in animal cells as in budding yeast, several studies suggest a role for the centrosome in cytokinesis. The most direct evidence for a role for centrosomes in cytokinesis has come from observations of cell division in mammalian tissue culture cells in which the centrosome was surgically removed, or in an acentriolar *Drosophila* cell line, which showed that a high frequency of these cells specifically failed to complete cytokinesis (Piel et al. 2001). These cells were able to complete cleavage furrow ingression and often remained connected by a narrow cytoplasmic bridge for an extended period of time. Unlike normal cells, these cells were not able to undergo abscission and separate this bridge and complete cytokinesis. As discussed earlier, these cells also failed to enter the next cell cycle. Observational studies are consistent with the centrosome playing an important role in cytokinesis. Using time-lapse video microscopy of cells stably expressing the centrosomal protein centrin fused to GFP as a centriole marker, Piel et al. observed that immediately before abscission, the mother centriole transiently and quickly migrates to the intracellular bridge near the midbody. Each centrosome is comprised of two distinguishable centrioles, a mother and daughter. Only when the mother centriole moved back from the bridge to the center of the cell did cytokinesis (abscission) finish. This is reminiscent of previous observations in which centrosomes in association with the Golgi complex, appear to shift localization from poles to the intracellular bridge and

back at the end of mitosis (Mack & Rattner 1993, Moskalewski & Thyberg 1992). Similarly, the recycling endosome-associated protein FIP3 localizes first to the centrosomes during anaphase, and then localizes to the midbody in telophase, where is required for cytokinesis/abscission (Wilson et al. 2004). These observations suggest that centrosomes and associated organelles must temporally and spatially come in close contact with the midbody for completion of cytokinesis to occur.

What function might this centrosome repositioning serve? Because Golgi complex associates with migrating centrosomes, this transient movement may deliver membrane and secretory vesicles required for complete cell separation. However another exciting possibility is that centrosomes harbor regulatory components required for mitotic exit and cytokinesis, as in budding and fission yeast. Recent studies are beginning to suggest that both ideas may be correct. Potential homologs of several SIN/MEN components have been identified (see Table 1). Several have been characterized (centriolin, Polo kinase, Cdc14A, Lats1,2 kinases) and shown to localize to centrosomes and play a role in cytokinesis (Carmena et al. 1998, Gromley et al. 2003, Kaiser et al. 2002, Mailand et al. 2002, McPherson et al. 2004, Yang et al. 2004). Centriolin, which is related to *S. pombe* Cdc11p and *S. cerevisiae* Nud1p, localizes to maternal centrioles and to the midbody (Gromley et al. 2003). Interestingly, these authors showed that centriolin could bind yeast Bub2p. Loss of function of Centriolin causes cells to have defects in abscission and remain connected for extended periods of time in a manner very similar to the phenotype of cells lacking centrosomes (Gromley et al. 2003). Centriolin depletion using RNAi also causes a G_1 arrest, as described above. A recent study showed that Centriolin may function to recruit factors required for targeted secretion to the midbody, which is required for abscission (Gromley et al. 2004). Interestingly, depletion of the centrosomal protein

Pericentrin/Kendrin (Gromley et al. 2003) or deletion of the Sid2p/Dbf2p homolog Lats2 (McPherson et al. 2004) also causes a similar cytokinesis defect. Together, these studies suggest that there may be a SIN/MEN pathway in mammalian cells that regulates cytokinesis; however, it has not yet been shown that these proteins function together as part of a signaling network as in yeast.

SIN/MEN HOMOLOGS AND CELL CYCLE CHECKPOINTS

Several laboratories have shown that cytokinesis failures induced by a number of different treatments cause cells to arrest in the G_1 phase of the following cell cycle as tetraploid cells (see Stukenberg 2004 for review). The arrest depends on p53 (Andreassen et al. 2001b). This arrest was initially termed a tetraploidy checkpoint (Andreassen et al.2001b), although recent results indicate that it may not be tetraploidy or cleavage failure that triggers the checkpoint (Uetake & Sluder 2004). What actually is monitored remains a mystery. Regardless of what triggers the arrest, it bears some interesting similarities to the cytokinesis checkpoint in fission yeast, which arrests cells as binucleate cells for a prolonged period following cleavage failure. It would be interesting to determine if any of the mammalian homologs of components required for the checkpoint in fission yeast function in this arrest in mammalian cells. Recent results suggest that this may be the case. Normally, cells treated with microtubule-depolymerizing drugs arrest in mitosis because of the spindle checkpoint. These cells eventually leak past this checkpoint, exit mitosis without undergoing cytokinesis, and arrest in G_1 phase of the next cell cycle in a p53-dependent manner. One report showed that after this treatment, the arrested cells express high levels of p53 consistent with it being required for the arrest (Iida et al. 2004). However, if the same treatment was done to cells overexpressing a dominant-negative form of the Sid2

[SIN pathway)/Dbf2 (MEN pathway)] homolog Lats1 (Warts), these cells failed to arrest in G_1 and induce p53. It has also been noticed that the mammalian Cdc14 homologs Cdc14A and Cdc14B bind p53 and dephosphorylate it at Ser315 (L. Li et al. 2000). This would likely stabilize p53 because phosphorylation at this site has been shown to promote degradation of p53 (Katayama et al. 2004). It will be interesting to see if Lats1/Warts kinase acts through Cdc14 to promote p53 stability and G_1 arrest following cleavage failure.

Studies in *Drosophila melanogaster* have shown that Warts functions together with a second kinase hippo, probably a homolog of mammalian Mst2 kinase, to prevent tumor formation (Harvey et al. 2003, Hay & Guo 2003, Pantalacci et al. 2003). Mst2 is related to the SIN kinases Sid1p and Cdc7p, as well as to budding yeast Cdc15p. Warts/Lats1 kinase has been shown to function as a tumor suppressor in mice as well (St. John et al. 1999). It will be interesting to determine whether Warts kinase and the Mst2 (hippo) kinase function as tumor suppressors by inhibiting cell cycle progression following cleavage failure. At present, these results suggest that SIN/MEN homologs may be required to promote p53-dependent arrest following cleavage failure, reminiscent of the cytokinesis checkpoint in *S. pombe*. This arrest is also bears similarity to the p53-dependent G_1 arrest following depletion of a number of centrosome proteins (see above) and perhaps the G_1 arrest induced by surgical removal of the centrosome (Hinchcliffe et al. 2001). Whether SIN/MEN homologs are involved in this arrest is unknown.

The Centrosome and Genotoxic Stress

Maintenance of genomic integrity is critical to normal development and disease prevention, and conserved DNA damage and replication checkpoints delay the cell cycle to allow repair of genetic lesions or completion of

Cytokinesis checkpoint: halts further rounds of nuclear division if cytokinesis is delayed

DNA replication. In systems ranging from mammalian tumors to early *Drosophila* embryos, checkpoint failures that allow DNA damage or incomplete replication to persist into mitosis triggers "mitotic catastrophe," a poorly understood process characterized by delays in metaphase followed by chromosome segregation and cytokinesis failures. The resulting cells then arrest in G_0 or die by apoptotic or nonapoptotic mechanisms (Andreassen et al. 2001a, Canman 2001, Roninson et al. 2001, Sibon et al. 2000). (The cellular and molecular basis of mitotic catastrophe is poorly understood, but this process appears to be a significant cause of chemotherapy-induced cell death in tumors and may serve an important genome maintenance function (reviewed in Roninson et al. 2001). Recent studies in *Drosophila* embryos and mammalian cultured cells indicate that mitotic catastrophe is linked to centrosome disruption, which may contribute to subsequent chromosome segregation and cytokinesis failures (Roninson et al. 2001). Recent studies in flies and human cells indicate that the Chk2 kinase is required for centrosome disruption on checkpoint failure, indicating that mitotic catastrophe is a genetically programmed response to genotoxic lesions (Takada et al. 2003). Chk2 is a human tumor suppressor, which raises the possibility that defects in damage-dependent centrosome disruption contribute to genomic instability and cancer progression.

Centrosome Inactivation in Early Embryos

Drosophila embryogenesis is initiated by 13 very rapid mitotic divisions that proceed without cytokinesis. These syncytial divisions, similar to the cleavage stage divisions in other embryos, are characterized by alternating S and M phases without intervening gap phases (Foe & Alberts 1983). The first 9 divisions take place in the interior of the embryo, but the majority of nuclei migrates to the cortex and form a monolayer by interphase of division 10. The final 4 syncytial blastoderm stage nuclear divisions (mitosis 10–13) take place in a cortical monolayer, and during these divisions the length of S phase progressively increases whereas M phase remains relatively constant. The DNA replication checkpoint is required to delay mitosis as S phase slows during these final syncytial blastoderm divisions (Sibon et al. 1997, 1999). As a result, embryos mutant for the replication checkpoint spontaneously initiate mitosis before S phase is completed, triggering mitotic catastrophe. Time-lapse confocal microscopic analyses show that checkpoint failure triggers mitosis-specific centrosome inactivation, anastral spindle assembly, and delays in mitosis, and centrosome inactivation correlate with loss of multiple components of the γTuRC from a core centrosome structure (Sibon et al. 2000). In wild-type embryos, identical mitotic defects are triggered by DNA replication inhibitors, a wide range of DNA damaging agents, and direct injection of restriction enzyme-digested DNA. Centrosome disruption and mitotic division failure thus appear to be a normal response to genotoxic lesions at the onset of mitosis (Sibon et al. 2000, Takada et al. 2003). Following division failure, the resulting nuclei drop into the interior of the embryo and are degraded. In syncytial embryos, mitotic catastrophe eliminates nuclei carrying DNA damage and thus serves a genome maintenance function analogous to apoptosis.

Mutations in the *Drosophila* homolog of Checkpoint kinase 2 (Chk2), encoded by the *mnk* gene, block all aspects of mitotic catastrophe in early embryos (Takada et al. 2003). The response is restored by a wild-type Chk2 transgene or injection of GST-Chk2 fusion protein, demonstrating that mitotic catastrophe, at least in fly embryos, is a genetically programmed response to genotoxic stress.

Centrosome Inactivation in Mammalian Cells

Mitotic catastrophe in mammalian cells is also triggered by G_2/M checkpoint failures and is

Centrosome inactivation: the reduction of the microtubule-nucleating proteins at the centrosome

characterized by delays in metaphase, chromosome segregation and cytokinesis failures, and cell death. In some cells, mitotic division failure is followed by apoptosis. However, cells more commonly arrest in G_1 or die by a nonapoptotic mechanism (Roninson et al. 2001). Cytologically similar mitotic catastrophe responses have been described in diverse systems, including primary mouse embryo fibroblasts, *Drosophila* embryos, and a number of cultured cells (Brown & Baltimore 2000, Bunz et al. 1998, Chan et al. 1999, Liu et al. 2000, Sibon et al. 2000) Significantly, mitotic catastrophe may be the primary mechanism of cell death in a number of tumor cells lines following treatment with chemotherapeutic agents (Roninson et al. 2001).

A hallmark of damage-induced mitotic catastrophe is accumulation of cells with large polyploid nuclei or multiple nuclei. In vivo studies in human colorectal tumor cells demonstrate that these cells can be formed by mitotic division failure(Bunz et al. 1998). Following ionizing radiation, HCT116 cells progress into mitosis and chromosomes align at the metaphase plate. However, anaphase chromosome segregation and cytokinesis fail, producing polyploid cells that contain bilobed nuclei (Bunz et al. 1998). Following division failure, nuclei fragment into compact masses that resemble clusters of grapes. Similar nuclear morphology is observed during apoptosis, and conventional apoptosis is sometimes observed following damage-induced division failure. However, cells produced by damage-induced mitotic failure are often TUNEL negative, and DNA isolated from these cells does not show laddering characteristic of apoptosis (Lock & Stribinskiene 1996, Nabha et al. 2002). In addition, in some cases cell death following is not blocked by apoptotic inhibitors, cells do not contract or bleb, and apoptotic bodies are not formed (reviewed by Roninson et al. 2001). Cell death by mitotic catastrophe thus appears to be distinct from apoptosis in both cell cycle phase and mechanism of execution.

Several recent studies have linked mitotic catastrophe to Chk2-dependent centrosome disruption, suggesting that a conserved signaling mechanism triggers this response. Hut et al. analyzed centrosomes as hamster cells progress into mitosis prior to completion of DNA replication (Hut et al. 2003), whereas Castedo et al. (Castedo et al. 2004b) analyzed γ tubulin distribution when interphase and mitotic cells are fused, driving the interphase nucleus into mitosis and bypassing the G_2/M checkpoint. Using GFP-γ tubulin as a centrosome marker, Hut et al. showed that centrosomes frequently fragment when checkpoint control is disrupted and mitosis is initiated before S phase is completed. These cells often assemble multi-polar spindles and progress through an aborted mitotic division to produce a single polypoid cell. These authors also found that cells carrying a mutation that disrupts DNA damage repair spontaneously show similar mitotic defects, indicating that centrosome fragmentation is not due to the caffeine treatments used in the replication studies. γ tubulin localization is also disrupted when G_2/M phase checkpoint control is bypassed by cell fusion (Castedo et al. 2004a,b). Significantly, this response requires Chk2 kinase (Castedo et al. 2004a,b), suggesting that active disruption of centrosome function in response to checkpoint failure is triggered by a conserved kinase pathway.

The timing of DNA damage may be critical to the mitotic response to DNA damage. Mikhailov et al. (2002) used laser light to induce DNA damage during prometaphase and found that these cells do not show centrosome defects. However, mitosis was delayed and H2Ax histone was phosphorylated, indicating that damage was present and detected by the cellular machinery. We have found that inducing DNA damage during mitosis has no clear effect on centrosome structure in early *Drosophila* embryos (S. Takada & W. Theurkauf, unpublished data), suggesting that centrosome disruption may require transit through the G_2/M transition with DNA

lesions. Mitotic catastrophe signaling may therefore require association of proteins with DNA lesions prior to mitotic chromosome condensation.

As discussed elsewhere in this review, a growing body of evidence links centrosome function to mitotic exit and cytokinesis. The observations on checkpoint-defective cells and embryos described above suggest that DNA lesions lead to centrosome inactivation, which lead to chromosome segregation and cytokinesis failures on mitotic exit. This mitotic catastrophe response eliminates damaged cells from the population. Inhibition of centrosome function by the Chk2 tumor suppressor, leading to mitotic exit defects, may therefore serve a function analogous to apoptosis in maintaining genome integrity. This pathway could have important implications for tumor suppression and chemotherapeutic treatment of cancer.

CONCLUSIONS

A growing body of evidence demonstrates that centrosomes and SPBs are involved in an increasing number of regulatory processes in cells. Centrosomes and SPBs provide a scaffold for binding numerous regulatory molecules. Among these are proteins involved in cell cycle progression and checkpoint control. Recent work shows that centrosomes are crucial for several cell cycle transitions, including entry into mitosis and progression from S phase to G_1. In budding and fission yeast, SPBs control exit from mitosis and progression through cytokinesis. Proteins involved in mitotic exit are sometimes positioned asymmetrically on the two SPBs. Centrosomes can also respond to cellular changes. For example, centrosomes lose their microtubule organizing activity and prevent mitosis when cells are exposed to genotoxic and other stresses. The ensuing failure in mitosis or cytokinesis results in polyploid cells that usually result in cell death, thus eliminating damaged cells from the population. The multitude of regulatory proteins that associate with centrosomes suggests that the number of regulatory processes in which centrosomes participate is only beginning to be revealed.

GLOSSARY

Centrioles: microtubule-based structures comprised of α/β tubulin and other proteins surrounded by pericentriolar material.

Centrosome inactivation: the reduction of the microtubule-nucleating proteins at the centrosome.

Cytokinesis checkpoint: halts further rounds of nuclear division if cytokinesis is delayed

Microtubule organizing center (MTOC): structures of diverse morphology that duplicate every cell cycle and nucleate the growth of microtubules.

Mitotic exit network (MEN): signaling network required for mitotic exit in the budding yeast *S. cerevisiae*.

Pericentriolar material (PCM): fibrillar material surrounding centrioles in the centrosome that nucleates the growth of new microtubules.

Septation initiation network (SIN): signaling network in *S. pombe*, analogous to MEN in *S. cerevisiae*, required for cytokinesis.

Spindle pole body (SPB): yeast equivalent of the centrosome.

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Chromosome Segregation and Aneuploidy series

Centrosome control of the cell cycle

Stephen Doxsey[1], Wendy Zimmerman[2] and Keith Mikule[1]

[1]Program in Molecular Medicine, University of Massachusetts Medical School, Worcester, Massachusetts 01605, USA
[2]Department of Molecular and Cellular Biology, Harvard University, Cambridge Massachusetts 02138, USA

Early observations of centrosomes, made a century ago, revealed a tiny dark structure surrounded by a radial array of cytoplasmic fibers. We now know that the fibers are microtubules and that the dark organelles are centrosomes that mediate functions far beyond the more conventional role of microtubule organization. More recent evidence demonstrates that the centrosome serves as a scaffold for anchoring an extensive number of regulatory proteins. Among these are cell-cycle regulators whose association with the centrosome is an essential step in cell-cycle control. Such studies show that the centrosome is required for several cell-cycle transitions, including G_1 to S-phase, G_2 to mitosis and metaphase to anaphase. In this review (which is part of the *Chromosome Segregation and Aneuploidy series*), we discuss recent data that provide the most direct links between centrosomes and cell-cycle progression.

Introduction

Chemical reactions in solution can be inefficient. In a multi-component biochemical reaction, the first component must locate, contact and modify its target before other steps can proceed. However, if all components of the reaction are physically linked together at a common site, the efficiency of the process can be enhanced. Perhaps the best example of such 'solid-state biochemistry' is the formation of signaling 'modules' in which multiple kinases are physically integrated in a way that facilitates a series of sequential binary interactions, thus creating a protein kinase cascade [1]. Mathematical modeling indicates that protein scaffolding can significantly increase the efficiency of kinase signaling pathways [2]. Physical linkage of molecules in a common pathway could increase the local concentration of components, limit nonspecific interactions and provide spatial control for regulatory pathways by positioning them at specific sites in proximity to cellular targets (e.g. other pathways, organelles, etc.) or to incoming signals from within or outside the cell. Scaffolding mechanisms could also provide temporal control of signaling events such as activation of cell-cycle transitions. In the process, the scaffold network could itself be monitored by its ability to ensure anchoring and functional outputs of regulatory pathways.

A growing body of evidence indicates that centrosomes serve as multiplatform scaffolds for a multitude of signaling networks. The centrosome in animal cells is usually located at the cell center, where it serves to nucleate polarized microtubule arrays for organizing cytoplasmic organelles and primary cilia in interphase cells, and for mitotic spindle organization and cytokinesis during mitosis. The centrosome is ~ 1–$2\ \mu m$ in diameter and consists of two barrel-shaped centrioles arranged perpendicular to one another, surrounded by the pericentriolar material (PCM). Estimates suggest that the centrosome comprises hundreds of proteins, including many large (200–450 kDa) coiled-coil scaffold proteins that serve as docking sites for a growing number of regulatory and other activities (Table 1; see also Supplementary Table S1 online) [3]. The PCM is in part organized by centrioles [4] and contains γ-tubulin ring complexes (γTuRCs), which nucleate microtubules, although other proteins also appear to be involved in this process [5]. Microtubule anchoring (distinct from nucleation) can occur at the distal appendages of the older or 'mother' centriole at least during some cell-cycle stages [6].

Table 1. Proteins reported to localize to the centrosome[a]

Category	Number of proteins per category
Ubiquitination and protein degradation	23
Nuclear transport/spindle assembly	4
Cytoskeletal regulators	22
CDKs and cyclins	5
Mitotic regulators	8
Chaperonins	3
Apoptosis related	8
DNA damage checkpoint	4
MAPK pathway	8
Spindle checkpoint	6
Mitotic exit/MEN	9
Cytokinesis/SIN	11
Transcription regulators	4
mRNA/mRNA processing	6
G_1/S regulation	2
Wnt signaling	3
Membrane receptor signaling	13
Other kinases/phosphatases	7
Golgi regulation	2
Other enzymes	10
Structural/scaffold proteins	60
Microtubule associated proteins (MAPS)	31
Motor proteins	15
Calcium binding	5
Other proteins	30
Viral proteins and infectious agents	13

[a]For a complete, extensively referenced, tabulation of the individual proteins, see Supplementary Table S1 online.

As many regulatory molecules are found at centrosomes, it is tempting to speculate that centrosomes serve as solid-state signaling machines capable of regulating many cellular functions, although, in most cases, the function of the centrosome-anchored fraction of these molecules has not been determined.

The substantial number of regulatory molecules that localize to the mammalian centrosome suggests the presence of complex regulatory networks at this site. For example, scaffold proteins such as the budding/fission yeast Nud1p/Cdc11p anchor multiple signaling molecules at the spindle pole body (the yeast centrosome equivalent) to control mitotic exit and cytokinesis [7]. In addition, many coiled-coil centrosomal proteins that act as scaffolds for anchoring protein kinases have been identified (e.g. protein kinases A, B and C) [8]. More recent results demonstrate a requirement for centrosomal anchoring of regulatory pathways in the control of cell-cycle progression (see below and Box 1). These observations provide some of the first functional links between centrosomes and regulatory networks and are the focus of this review. We discuss recent studies, primarily in mammalian cells, that provide the most direct evidence for a link between centrosomes and cell-cycle progression from G_1 to S-phase (G_1–S), G_2 to M-phase (G_2–M) and metaphase to anaphase (M–A); centrosomal regulation of cytokinesis has been reviewed recently [9].

The centrosome in the G_1–S transition
Removal of core centrosome components
Studies designed to remove centrioles and associated PCM from cells by microsurgical cutting [10] or laser ablation [11] have provided direct evidence for centrosomes in cell-cycle progression (Figure 1a,b). Removal of core centrosome components resulted in the formation of acentriolar microtubule organizing centers (MTOCs) containing several PCM proteins [11,12], similar to those of higher plants and some meiotic systems [13,14]. The animal cells containing acentriolar MTOCs formed functional mitotic spindles, but about half failed to cleave into two daughter cells during cytokinesis. All cells with acentriolar MTOCs, whether they completed cytokinesis or failed (forming tetraploid cells), did not initiate DNA replication (BrdU-negative, Figure 1a). Moreover, ablation of one of two centrosomes in prometaphase cells produced a centrosome-containing daughter that continued to cycle (BrdU-positive) and a daughter cell with an acentriolar MTOC that did not enter S-phase (BrdU-negative, Figure 1b). By contrast, extra centrosomes (or nuclei) created by cell-fusions or by inhibition of cytokinesis using actin-perturbing drugs, did not inhibit cell-cycle progression [15,16]. In addition, cell-cycle progression did not appear to require centrosome-associated microtubules. Normal cycling diploid cells progressed through G_1 without microtubules (after nocodazole treatment), suggesting that they were not required for this cell-cycle transition.

Box 1. Coordinating cycles: cell, centrosome and DNA cycles

Accurate cell division requires the coordinated completion of three separate but interdependent cycles namely, the cell, centrosome and nuclear cycles [42–44] (Figure I). However, recent reports [24,29] have suggested that both the nuclear and cell cycles depend upon the centrosome or centrosome cycle for advancement.

The cell cycle
The cell, or cytoplasmic, cycle consists of the sequential activation and deactivation of cyclin-dependent kinases (CDKs). Control is provided through the availability of partner cyclins (cyc) and by phosphorylation/dephosphorylation events. CDK inhibitors (CKIs) provide a third level of CDK regulation by binding to and inactivating CDK–cyc complexes. p53 family members and other proteins transcriptionally regulate CKI levels. CKIs are upregulated in response to signaling pathways that monitor nutrient availability (i.e. serum), osmolarity/salinity, temperature, DNA damage and other parameters and serve to arrest the cell cycle [45].

The centrosome cycle
Following cytokinesis, a normal diploid cell inherits one centrosome with two centrioles that replicates during S-phase, separates around G_2–M, and becomes part of the spindle poles during M phase. The molecular details of centrosome duplication are unclear. However, most researchers would agree that duplication is initiated at the G_1–S transition and is coincident with Cdk2-dependent phosphorylation of centrosome substrates and the subsequent moving apart or 'splitting' of the centriole pair (blue/red cylinder) [23,46]. Daughter centrioles then arise from the side of each centriole on or near the pericentriolar material (PCM) and become mature full-length structures by the end of G_2. By M-phase both centrosomes have acquired the maximal amount of PCM.

The nuclear cycle
During each cell-division cycle, the genome must be duplicated, condensed and precisely divided among daughter cells. Approximately at G_1–S, Cdk2 phosphorylation of the origin of replication (ORC)-bound pre-replication complex initiates DNA polymerase recruitment and firing of the origins [44], followed by complete genome replication in S-phase. In G_2, the nucleotide excision repair complex detects DNA mismatches or strand breaks and halts the cell cycle through checkpoint kinase activation, so that repairs can be completed before entry into mitosis. At the end of G_2, Cdk1 activation initiates nuclear envelope breakdown and chromosome condensation, two hallmarks of mitotic entry. Nuclear lamin phosphorylation, along with microtubule ingression are responsible for nuclear envelope breakdown [43], whereas chromosome condensation requires histone H3 phosphorylation [47]. Mitosis proceeds with chromosome alignment on the metaphase plate, separation of sister chromatids at anaphase and cytokinesis.

Linking cycles
The use of common regulatory complexes, such as CDKs, to coordinate the cell, centrosome and nuclear cycles is one way of coupling them. Another method of coordination is accomplished by localizing complexes to a given site. This occurs at the centrosome at both the G_1–S and G_2–M transitions (large red arrows; see also Table 1). At G_1–S, cycE recruitment to the centrosome is needed for DNA replication [24], whereas Cdk2 activity is required at the centrosome to start its cycle [23]. At G_2–M, centrosome-bound Cdk1 is activated first and initiates mitosis [29]. These strategies not only provide a template for cell-cycle activation at certain key stages but, in the process, could serve to monitor the integrity of the centrosome (at least the binding sites for cell-cycle regulatory molecules).
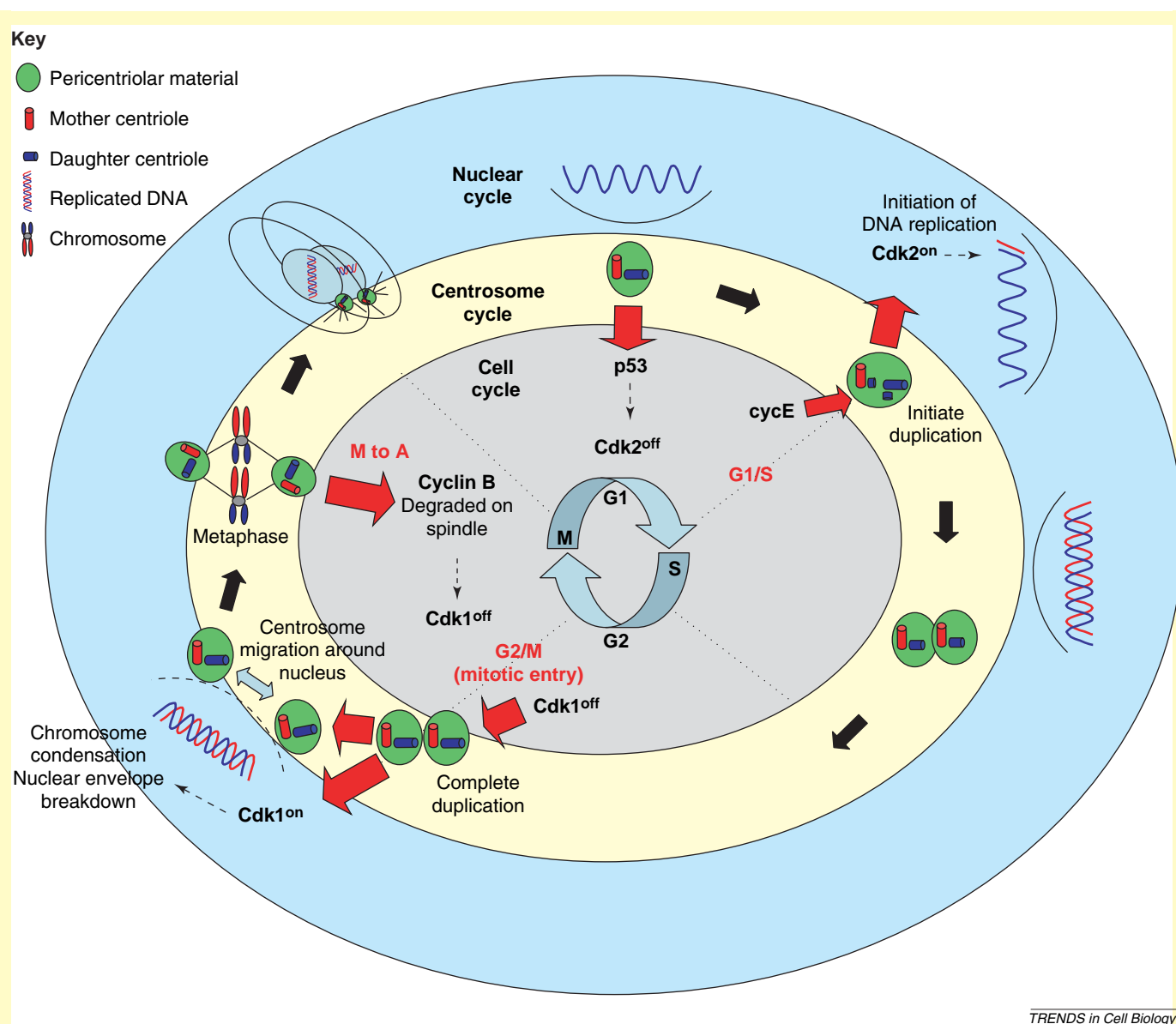
Figure I. Centrosome-associated steps during the cell cycle. Only the cell-cycle transitions that appear to require centrosomes are shown (red arrows).

By contrast, cells arrested within G_1 in the presence of taxol or in nocodazole after failed cytokinesis [17,18]. However, results from these experiments are difficult to interpret as stabilized microtubules in taxol-treated cells and the consequences of failed cytokinesis might influence cell-cycle progression.

Changes in centrosome protein expression levels or localization induce G_1 arrest

Molecular studies have also uncovered a role for individual centrosome components in cytokinesis and cell-cycle progression (Figure 1c,d). Centriolin is a component of the mother centriole that shares homology with Nud1p and Cdc11p [19], budding and fission yeast proteins involved in cytokinesis/mitotic exit, respectively [9]. Centriolin depletion or overexpression of the Nud1p/Cdc11p homology domain delayed cytokinesis for extended periods of time. Following the cytokinesis delay, cells did not progress into S-phase but remained in the G_1 peak when

examined by flow cytometry (2N DNA content). AKAP450 is a protein of the PCM with a C-terminal domain that serves a centrosome targeting function [20,21]. Ectopic expression of the AKAP450 C-terminus mislocalized endogenous AKAP450 and protein kinase A (PKA) from centrosomes and induced cytokinesis defects and G_1 arrest. Thus, the results from both centrosome protein perturbation and centrosome/centriole removal studies suggest that G_1 arrest could be a consequence of prior cytokinesis defects.

Does mitotic dysfunction cause G_1 arrest?

The central role of centrosomes in mitotic spindle organization and cytokinesis suggests that mitotic dysfunction leads to G_1 arrest. However, recent studies indicate this might not be the case. Microinjection of antibodies against PCM-1 into early interphase mouse zygotes prevented cell-cycle progression into S-phase [22]. In another study, over 20 proteins found at five distinct

(a) Surgical removal

Interphase

Micro-needle

Mitosis   Failed mitosis

G₁ arrest   G₁ arrest

(b) Laser ablation

Prometaphase

Laser

Mitosis, cytokinesis defects

G₁ arrest   Cycling

(c) siRNA depletion

Interphase

PCM   Centriole

Mitosis

G₁ arrest   G₁ arrest

(d) Protein expression

Microinject cDNA encoding CLD

G_1

G₁ arrest

(e)

(i) Cyclin E — Centrosome localization domain (CLD)
Express cyclin E → Cyclin E Binds centrosome Centrosome → G_1 → S-phase Accelerates S-phase entry

Cdk2 ✗ Cyclin E
Cyclin E mutations (blue) that block Cdk2 binding still accelerate of S-phase entry

(ii) Cyclin E
Express cyclin E w/o CLD → Cyclin E No centrosome binding Centrosome → G_1 → S-phase Normal S-phase timing

(iii) Cyclin E — Centrosome
Express centrosome localization domain (CLD) → Cyclin E Centrosome → G_1 → S-pha se
Endogenous cyclin E lost from centrosome
Mutant CLD that does not bind to centrosomes had no effect on cell-cycle progression

Conclusion. Cyclin E has a centrosome binding domain essential for promoting S-phase entry in a Cdk2-independent manner

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regions of the centrosome (Figure 1c,d) were individually targeted for depletion by siRNAs. Many showed no detectable change in spindle function, cytokinesis or microtubule organization, but nearly all induced G_1 arrest, as shown by accumulation in the 2N peak by flow cytometry, lack of BrdU incorporation and reduction in the activity of cyclin-dependent kinase2–cyclinA/E complexes (Cdk2–cycA/E) (K. Mikule, S. Doxsey and P. Kaldis, unpublished). G_1 arrest was 'rescued' by returning targeted centrosome proteins to normal levels. These results suggested that cell-cycle arrest could be induced through discrete alterations in centrosome composition.

To more directly test the role of mitotic dysfunction in G_1 arrest, post-mitotic cells (early G_1) were microinjected with a plasmid encoding the shared centrosome-targeting region of pericentrin and AKAP450 that mislocalizes both proteins from centrosomes [20]. This significantly reduced the centrosome-bound fraction of endogenous pericentrin and prevented cells from entering S-phase (K. Mikule and S. Doxsey, unpublished). Consistent with these results are recent data showing that ablation of centrosomes in post-mitotic cells inhibited progression through the cycle (14/16 cells, A. Khodjakov, pers. commun.). Results from multiple approaches, from removal of entire centrosomes to depletion of single centrosome proteins, show that G_1 arrest can be induced from within G_1. In summary, the mitotic functions of the centrosome do not appear to contribute to the G_1 arrest induced by centrosome protein depletion or mislocalization.

Mechanism of centrosome-induced G_1 arrest

The molecular pathway leading to G_1 arrest induced by centrosome disruption is currently unknown. It is known that Cdk2–cycE/A complexes are required for initiating both centrosome duplication and entry into S-phase [23]. Recent results have demonstrated that cycE localizes to centrosomes and that overexpression of the cycE centrosome localization domain (CLD) disrupted centrosome binding of endogenous cycE (and cycA) and prevented entry into S-phase (Figure 1e) [24]. Ectopically expressed cycE accelerated entry into S-phase even with mutations that abolished CDK binding, but not with a mutation in the CLD. The authors suggested that centrosome targeting of cycE is essential for promoting S-phase entry in the absence of Cdk2 activity and that its loss during centrosome removal (and potentially centrosome protein depletion) might induce G_1 arrest. Interestingly, these results leave open the possibility that the centrosome might control S-phase entry and therefore the nuclear cycle (Box 1) through mechanisms such as cycE binding that are independent of the cell cycle (Cdk2 activity). It

remains to be determined whether centrosomes depleted of individual components by siRNA or acentriolar MTOCs produced by centrosome/centriole removal have lost their interaction with these cyclins or whether other changes in centrosomes trigger G_1 arrest (see Box 2).

Activation of a centrosome-induced G_1 checkpoint?

Evidence suggests a role for p53 in sensing the centrosome-induced G_1 checkpoint. Centrosome protein depletion-induced G_1 arrest was suppressed in tumor cells with abrogated p53 function and in cells acutely depleted of p53 by siRNAs [25]. Probing pathways upstream of p53 with inhibitors or siRNAs identified the p38 stress-activated signal-transduction pathway as a contributor to the G_1 arrest. p38 is also found associated

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Box 2. Models for G_1 arrest

The mechanism of cell-cycle arrest following centrosome perturbation is unknown. Below, we present three speculative models that might account for this arrest.

Centrosome disruption

We favor a model in which centrosome disruption at the structural (centrosome removal) or molecular level (loss of centrosome-associated protein) triggers G_1 arrest. This model can account for changes in the structure of the centrosome or microtubule-organizing center (MTOC) induced by both physical and molecular disruption. Assuming that long elastic coiled-coil centrosomal proteins are exquisitely interconnected, disruption of one protein could dramatically change the overall molecular organization of the centrosome. Centrosome-anchored regulatory molecules or pathways that control cell-cycle progression could be modified through loss of, or failure to release/activate, a positive signal [24]. Alternatively, molecular changes could be sensed directly as 'damage' or disruption, leading to a stress response, perhaps involving molecular chaperones, and subsequent cell-cycle arrest.

Disruption of a common centrosome function

Another possible cause of cell-cycle arrest is that all centrosome perturbation conditions alter a common centrosome-associated function. Caveats of this idea are that the common function would need to be perturbed by depletion of over 15 individual centrosome proteins and occur post-mitotically, within G_1 of the cell cycle.

Centrosome nuclear shuttling

Given the precedence for centrosome proteins localizing to/functioning in the nucleus (e.g. centrin) [21,48] and nuclear proteins localizing to/functioning at the centrosome (e.g. Orc2, Orc6, RAD51) [49–51], it is possible that centrosome–nucleus shuttling could be used to create obligate steps at one organelle that are required for the function or cycling of the other. Alternatively, nuclear and centrosome duplication cycles could be coupled by localizing components of the pathways to two separate sites (nucleus, centrosome) so that both sites can be monitored before and during the cycles (e.g. Cdk2, cycA/E). A caveat of this model is that there are only a few proteins that show dual localization/function to nucleus and centrosome.

Figure 1. Role of centrosomes in G_1-to-S-phase progression. (a,b) Centrosome removal by cutting in interphase cells **(a)** or by laser ablation in prometaphase cells **(b)** ultimately gives rise (bottom row) to cells with a loosely focused microtubule array (yellow) organized by a microtubule-organizing center (MTOC) that lacks centrioles (red) but contains components of the PCM (purple) and perhaps other centrosome proteins. Nucleus, blue. Cells with acentriolar MTOCs arrest in G_1, whereas cells that have an intact centrosome continue to cycle [(b), right]. Mitosis is not dramatically altered, although cytokinesis defects are observed in both **(a)** and **(b)**. **(c,d)** siRNA-mediated depletion of centrosome-associated proteins or mislocalization of centrosome proteins by expression of the centrosome-localization domain (CLD) from injected cDNAs (e.g. AKAP450) induces G_1 arrest (bottom row). Cell-cycle arrest is observed upon depletion of proteins of the PCM (purple to white), centrioles (red to white) or other centrosomal structures (not shown). **(e)** Wild-type cyclin E with an endogenous CLD (i), but not a mutated CLD (ii), accelerates S-phase entry when overexpressed. A cyclin E mutant that lacks Cdk2 binding accelerates S-phase entry, like the wild-type protein (i). Expression of the wild-type CLD domain (iii), but not a mutant form that does not bind to the centrosome (iii), blocks entry into S-phase.

with the centrosome, and functional abrogation of p53, p38 or other members of these or other pathways involved in centrosome-associated G_1 arrest might explain why some cells continue to cycle despite the absence of centrioles [4,6].

G_1 arrest in cells with reduced centrosome protein levels fulfils the criteria for a checkpoint. The cells arrest at a specific cell-cycle stage until the process is 'repaired' by returning target protein levels to normal; it can be suppressed by inactivation of molecules in the checkpoint pathway (p53, p38). These data also suggest a mechanism whereby tumor cells with abrogated p53 or p38 pathways could avoid checkpoint activation and propagate centrosome defects and their downstream consequences (mitotic dysfunction and aneuploidy; Box 3).

The centrosome in the G_2 to M transition (mitotic entry)

A role for the centrosome in mitotic entry was suggested by early work showing that centrosomes induced progression into mitosis when injected into G_2-arrested starfish oocytes [26], and they activated maturation promoting factor (MPF), now known as Cdk1–cycB and accelerated mitotic entry in *Xenopus* eggs [27]. Subsequent work demonstrated that mitotic kinases and cyclins were present at centrosomes (see Table 1).

A role for centrosomal Cdk1 activation in the G2–M transition

More recent work has revealed a role for the centrosome in the G_2 to M transition in mammalian cells (Figure 2). Cdk1–cycB activation is a key event in initiating mitosis. Although cycB1 is present throughout the cytoplasm before prophase, active Cdk1–cycB1 was first detected at the centrosome during prophase and before phosphorylation of heterochromatic histone H3 in the nucleus [28]. This observation led the authors to suggest that centrosomes might function as sites of integration for proteins that trigger mitosis.

Drawing on this observation, Lukas and colleagues used a clever centrosome-targeting strategy to provide evidence that mitotic entry requires centrosome localization of Cdk1 and its modulators [29] (Figure 2). They showed that the checkpoint kinase 1 (Chk1) was present on centrosomes in interphase, but not mitotic cells, and that it acted as an inhibitor of Cdk1 activity. In fact, chemical inhibition of Chk1 activated centrosome-associated Cdk1 and induced premature entry into mitosis, as shown by increased microtubule nucleation, recruitment of the mitosis-specific motor Eg5 to spindles, premature separation of centrosomes and increased numbers of mitotic cells.

To test the role of centrosome localization in Chk1 function, the centrosome-localization sequence (CLS) of the centrosome protein AKAP450 was fused to wild-type and kinase-dead versions of Chk1 (wtChk1 and kdChk1). Expressed CLS-tagged wtChk1 was immobilized at the centrosome and was unable to phosphorylate substrates in the nucleus, whereas Chk1 without the CLS did so effectively. Forced immobilization of kdChk1 to centrosomes induced premature Cdk1 activation and premature mitotic entry, as seen with Chk1 inhibitors (above). By contrast, centrosome-targeted wtChk1 inhibited activation of Cdk1 at centrosomes, leading to mitotic failure and formation of polyploid cells with multiple centrosomes.

Inhibition of Cdk1 activity by Chk1 was not direct but was linked to inhibition of the Cdk1-activating phosphatase, Cdc25B [29]. Activation of Cdk1 through Cdc25B appeared to occur through centrosome-localized Chk1 [29,30]. Aurora-A kinase is required for recruitment of Cdk1–cycB to centrosomes and, thus, for activation of the kinase [31]. While not directly linked to Cdk1 activation, the centrosome-associated mitotic regulator Polo kinase (Polo) is also involved in mitotic entry. Centrosome localization of Polo at G2–M depends upon its C-terminal domain (polo box) [32–34]. Overexpression of this domain prevents mitotic entry, arresting cells with a 4N DNA content [32].

Box 3. Centrosomes, cell cycle, aneuploidy and tumorigenesis

Centrosome abnormalities and aneuploidy are hallmarks of most human cancers and animal models (Figure I) [52,53]. Moreover, centrosomes can play a causal role in generating aneuploidy through multipolar spindle formation **(a,b)** and chromosome missegregation **(c–e)**. The centrosome has the potential to disrupt genomic fidelity not only through mechanical changes in spindle organization, cytokinesis and chromosome segregation but also through its potential to disrupt centrosome-associated cell-cycle regulatory molecules, many of which are themselves implicated in tumor progression [45]. In this context, centrosome perturbations could lead to unscheduled cell-cycle events, which could lead to genetic instability.

Potential mechanisms of centrosome-associated aneuploidy

p53 In normal diploid cells, cells with centrosome defects undergo G_1 arrest in a p53-dependent manner. In p53-deficient human cancers, cells can continue to cycle, allowing centrosome defects to promote spindle defects **(b)**, improper chromosome segregation **(c–e)** and genetic instability. Similarly, cells that experience cytokinesis defects and subsequent cell-cycle arrest following disruption of specific centrosome proteins [19,21] can continue to cycle in p53-deficient cells, generating polyploid cells with supernumerary centrosomes.

G_2/M Centrosome disruption during the G_2–M transition could prevent Cdk1 activation or induce premature activation of the kinase. Centrosomal loss of Cdk1 activators (e.g. Cdc25B) or inability to release Cdk1 inhibitors from this site (e.g. Chk1) could prevent activation of Cdk1. Cells that arrest in G2 for extended periods might undergo mitotic failure before chromosome segregation, forming polyploid cells with extra centrosomes [29].

Metaphase to anaphase Centrosome disruption during the metaphase-to-anaphase transition could mislocalize scaffold proteins that constrain movement of cyclin B degradation machinery from spindle poles [37,39]. This could allow premature movement of degradation activity up the spindle to the chromosomes, leading to premature chromosome separation and aneuploidy.

G_1 to S Mislocalization of cyclin E binding from centrosomes through loss of putative centrosome scaffold proteins would be expected to block entry into S-phase [24]. In tumor cells deficient for p53 or other pathways that control entry into S-phase, cells might enter S-phase and continue to cycle despite the presence of a disrupted centrosome (loss of cycE scaffold protein). Alternatively, altered centrosomes might prematurely recruit cycE, which could force entry into S-phase and promote proliferation. This would be consistent with cycE overexpression seen in some tumors [54].
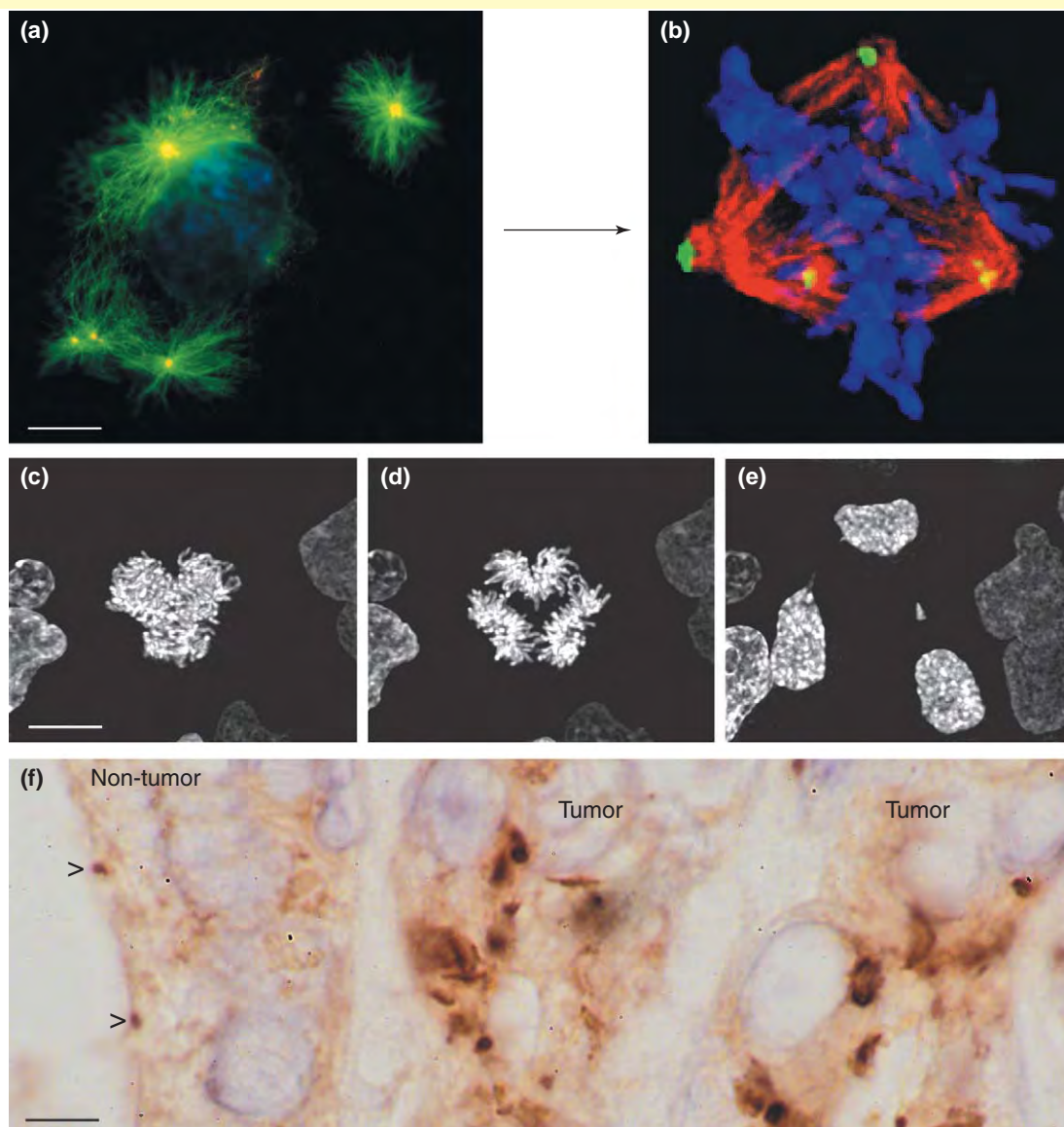
Figure I. Centrosome abnormalities, chromosome missegregation and aneuploidy in human tumor cells and tumors. **(a,b)** Excess centrosomes in tumor cells form multiple asters in interphase **(a)** and multipolar spindles **(b)** in mitosis. **(a)** Green, microtubules; yellow, centrosomes; blue, DNA. **(b)** red, microtubules; green, centrosomes; blue, chromosomes. **(c–e)** Multipolar spindles missegregate chromosomes **(c,d)** into three daughter cells **(e)**, inducing aneuploidy. Note the presence of chromosome(s) that do not get incorporated into the nucleus [**(e)**, center]. Centrosome abnormalities in a section of a human prostate tumor **(f)**. Normal prostate gland (left) showing a single centrosomes/cell (brown dots) stained with antibodies to the centrosome protein pericentrin (nucleus, blue). Cells in tumor glands have centrosomes with enlarged diameters, elongated forms and multiple copies (right). Bars, (a) 8 μm, (c) 12 μm, (f) 7 μm.

Collectively, these results indicate the presence of a cell-cycle-regulatory module in which positive and negative pathways are integrated at the centrosome to control mitotic entry. Because removal of centrosomes/centrioles does not prevent entry into mitosis (see above) [10,11], it would seem that centrosomal regulation of Cdk1 activation by Chk1 might not be an absolute requirement for mitotic entry. However, the PCM and other material in the acentriolar MTOC that is organized following centrosome removal might be sufficient for localization of kinases required for mitotic entry.

A role for centrosomal γ-tubulin in the G2–M transition

γ-Tubulin ring complexes (γTuRCs) are microtubule-nucleating complexes anchored at centrosomes in part by the centrosomal scaffolding protein pericentrin [35].

Disruption of the pericentrin–γTuRC interaction by peptides encoding the pericentrin γTuRC-binding domain or by siRNA-mediated pericentrin depletion induced arrest at G_2–M, followed by apoptosis in many cell types. Cells that failed to arrest revealed downstream consequences of pericentrin–γTuRC disruption, including loss of centrosomal γ-tubulin and astral microtubules. Additional work will be required to determine whether loss of centrosomal γ-tubulin mislocalizes cell-cycle-regulatory proteins from centrosomes (previous section) or changes other parameters that could lead to activation of a G_2–M checkpoint.

The centrosome/spindle pole in the metaphase-to-anaphase transition

The metaphase-to-anaphase transition is controlled in part by the spindle-assembly checkpoint that monitors
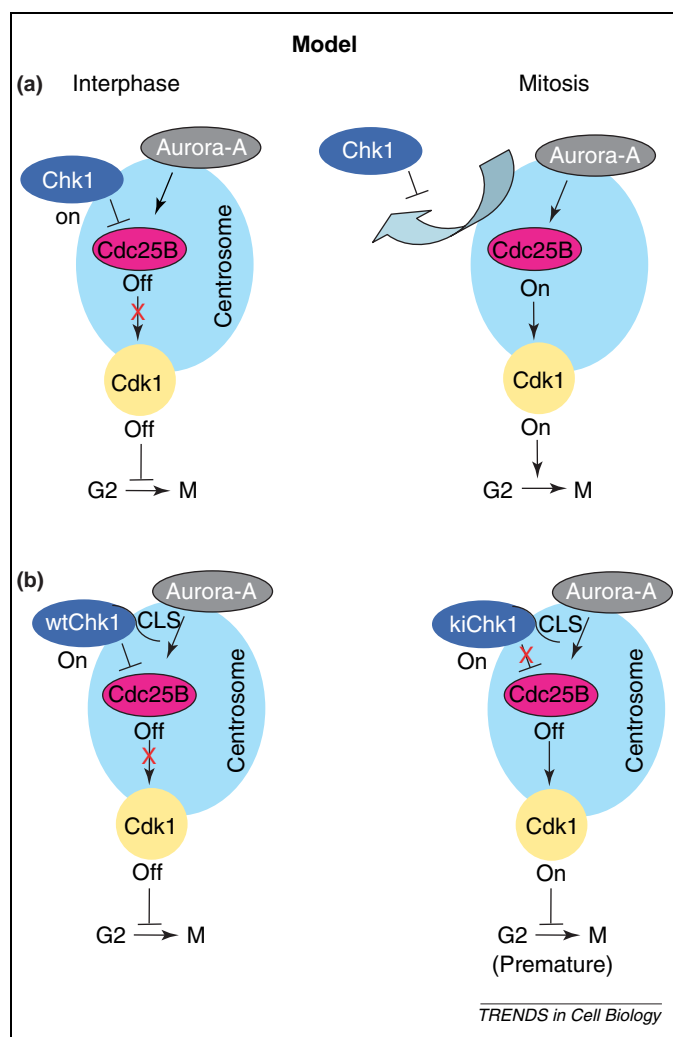
Figure 2. Role of centrosomes in G2 to M-phase progression. **(a)** Model for centrosome-bound-Cdk1 activation (see text). In interphase cells (left), Chk1 inhibits Cdk1 at the centrosome through inactivation of the Cdk1-activating phosphatase Cdc25B; G_2–M progression is prevented. In mitosis, Chk1 is lost from centrosomes (blue arrow), leading to activation of Cdk1 through Cdc25B, which is itself activated by Aurora A kinase (right); cells enter mitosis (right). **(b)** Wild-type Chk1 with a centrosome localization sequence of AKAP450 (CLS) localizes to centrosomes, inhibits Cdk1 activation at this site and prevents G_2–M progression (left), whereas kinase-inactive Chk1 with the CLS does not inhibit Cdk1, leading to premature entry into mitosis (right).

attachment of spindle microtubules to kinetochores of the chromosomes. Once the spindle-assembly checkpoint is satisfied, degradation of cyclin B (cycB), and cohesins that bind to sister chromatids, is initiated. While the importance of the kinetochore as a molecular scaffold for sensing and regulating the metaphase-to-anaphase transition has been extensively documented and is well accepted [36], a role for the spindle pole as a scaffold in regulating this transition has been largely neglected.

The first clear indication that the centrosome/spindle pole was involved in regulation of the M–A transition came from real-time observations of cycB–GFP destruction in *Drosophila* embryos [37]. Destruction of cycB–GFP (and the endogenous protein) at the M–A transition was spatially controlled, starting at the spindle pole and spreading up the spindle to the chromosomes. Moreover, in a *Drosophila* mutant that loses centrosome/spindle attachment ('centrocentrosomes fall off', cfo), cycB destruction occurred on

unattached centrosomes but not on the spindle, resulting in anaphase arrest [38]. Recent work in cellularized *Drosophila* embryos showed that two activators of the anaphase-promoting complex that degrade cycB, Fzy/Cdc20 and Fzr/Cdh1 [39], modulate cycB destruction. While both APC activators localized to centrosomes, Fzy/Cdc20 was responsible for the spindle-associated wave of cycB destruction, while Fzr/Cdh1 was required for destruction of cytoplasmic cycB; in syncitial embryos, only Fzy/Cdc20 is present.

Other studies have implicated the centrosome proteins γ-tubulin and the human pericentrin B homolog, Pcp1p, in regulation of the M–A transition. In *Aspergillus nidulans*, a cold-sensitive γ-tubulin allele did not inhibit spindle formation at the restrictive temperature but significantly delayed the M–A transition and failed cytokinesis [40]. Similarly, a mutation in Pcp1p of *Schizosaccharomyces pombe* inhibited the M–A transition without disrupting bipolar spindle assembly [41]. These studies, and those showing a role for pericentrin in the G_2–M transition, suggest that pericentrin homologs and γ-tubulin complexes at the centrosome might be involved not only in microtubule nucleation and anchoring but in regulation of multiple cell-cycle transitions.

Concluding remarks

The conventional picture of the centrosome as being under cell-cycle control is changing. The emerging picture is that the centrosome can exert control over the cell cycle. This suggests that the interrelationship between the centrosome and cell cycle might be required for transitions between several cell-cycle stages. By providing a scaffold for cell-cycle regulators and their activity (e.g. Cdk2, Chk1), the centrosome can influence cell-cycle progression (G_2 to M). Centrosome association of other cell-cycle regulators (e.g. cycE) appears to control cell-cycle progression (G_1 to S) without affecting the activity of their associated regulatory kinases (e.g. Cdk2). The requirement for intact centrosomes for progression from G_1 to S-phase suggests an important role for centrosomes in this transition, although the molecular mechanism has yet to be uncovered. We are at the beginning of understanding how centrosomes influence cellular regulation. Based on the growing number of centrosome-associated regulatory molecules, it is likely that there are many regulatory functions at centrosomes that await discovery. One particularly enticing idea is that the centrosome might anchor signal-transduction pathways and serve as a central site that receives and integrates signals from outside the cell and facilitates the conversion of these signals into cellular functions in the cell interior.

Supplementary data

Supplementary data associated with this article can be found at doi:10.1016/j.tcb.2005.04.008

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Membrane Type-1 Matrix Metalloproteinase (MT1-MMP) Exhibits an Important Intracellular Cleavage Function and Causes Chromosome Instability*

Vladislav S. Golubkov‡, Sarah Boyd§, Alexei Y. Savinov‡, Alexei V. Chekanov‡,
Andrei L. Osterman‡, Albert Remacle‡, Dmitri V. Rozanov‡, Stephen J. Doxsey¶,
and Alex Y. Strongin‡‖

From the ‡Cancer Research Center, The Burnham Institute, La Jolla, California 92037, the §School of Computer Science and Software Engineering, Monash University, Melbourne, Victoria 3800, Australia, and the ¶University of Massachusetts Medical School, Worcester, Massachusetts 01605

Elevated expression of membrane type-1 metalloproteinase (MT1-MMP) is closely associated with malignancies. There is a consensus among scientists that cell surface-associated MT1-MMP is a key player in pericellular proteolytic events. Now we have identified an intracellular, hitherto unknown, function of MT1-MMP. We demonstrated that MT1-MMP is trafficked along the tubulin cytoskeleton. A fraction of cellular MT1-MMP accumulates in the centrosomal compartment. MT1-MMP targets an integral centrosomal protein, pericentrin. Pericentrin is known to be essential to the normal functioning of centrosomes and to mitotic spindle formation. Expression of MT1-MMP stimulates mitotic spindle aberrations and aneuploidy in nonmalignant cells. Volumes of data indicate that chromosome instability is an early event of carcinogenesis. In agreement, the presence of MT1-MMP activity correlates with degraded pericentrin in tumor biopsies, whereas normal tissues exhibit intact pericentrin. We believe that our data show a novel proteolytic pathway to chromatin instability and elucidate the close association of MT1-MMP with malignant transformation.

Matrix metalloproteinases (MMP(s))[1] are a comprehensive family of zinc-enzymes that degrade the extracellular matrix and cell surface molecules (1). Understanding the function of these enzymes in carcinogenesis is critical for the design of anti-cancer pharmaceuticals (2). MT1-MMP is a prototypic member of the membrane-tethered MMP subfamily (3). A transmembrane domain and a cytoplasmic tail (CT) of MT1-MMP associate this abundant membrane-tethered protease with discrete regions of the plasma membrane and the intracellular milieu, respectively. Although MT1-MMP is present in normal tissues, its enhanced expression, unlike of any other of the 23 known human MMPs, is closely associated with aggressive, invasive malignancies (1, 3–5). MT1-MMP transgenic mice displayed mammary gland abnormalities and tumor promotion in mammary gland (6).

MT1-MMP functions as one of the main mediators of proteolytic events on the cell surface, and it is directly involved in the pericellular proteolysis of the extracellular matrix, cell surface adhesion, and signaling receptors and in the activation pathway of soluble secretory MMPs (5, 7–9) Cell surface-associated MT1-MMP acts as a growth factor in malignant cells and assumes tumor growth control (4). The conditional expression of MT1-MMP can, by itself, confer tumorigenicity on nonmalignant epithelial cells and cause the formation of invasive tumors (10). MT1-MMP also plays an important role in normal development; MT1-MMP knock-out mice are dwarfs, and they die prematurely (8, 11). A loss of the structurally similar primordial At2-MMP induces dwarfism in *Arabidopsis* plants (12). There is no extracellular matrix in plants, however, that is similar to the collagenous extracellular matrix of mammals. This datum alone is enough to suggest that the protease plays a role in certain functionally relevant intracellular events in addition to its role in pericellular proteolysis.

MT1-MMP is tightly regulated at the transcriptional and posttranscriptional levels both as a protease (through activation and inhibition) and as a membrane protein (via trafficking, internalization, and recycling) (13–15) The trafficking and the internalization, *via* clathrin-coated pits and caveolae, have emerged as the essential mechanisms that regulate the biological function of MT1-MMP (16–23). These new data, combined together, provided a compelling argument to investigate the trafficking and the intracellular compartmentalization of MT1-MMP in greater detail. These data also argue that there is a role for the protease in intracellular events in addition to its role in pericellular proteolysis.

Here, we have discovered compelling evidence that MT1-MMP is trafficked along the tubulin cytoskeleton. A fraction of cellular MT1-MMP accumulates in the centrosomal compartment. In the pericentrosomal compartment, active, functionally potent MT1-MMP degrades an integral centrosomal protein, pericentrin. Pericentrin is essential to the normal functioning of centrosomes in the mitotic spindle formation. MT1-MMP proteolysis of pericentrin causes chromosome instability, which is an early predictor of carcinogenesis. Overall, our results suggest an intracellular function for the membrane-tethered protease and an important role of MT1-MMP in the transition of cells from normalcy to malignancy.

‖ To whom correspondence should be addressed: Cancer Research Center, The Burnham Institute, 10901 North Torrey Pines Rd., La Jolla, CA 92037. Tel.: 858-713-6271; Fax: 858-646-3192; E-mail: strongin@burnham.org.

[1] The abbreviations used are: MMP, matrix metalloproteinase; MT1, membrane type-1; CT, cytoplasmic tail; MDCK, Madin-Darby canine kidney; siRNA, small interfering RNA; GFP, green fluorescent protein; PoPS, prediction of protease specificity; PDX, α1-anti-trypsin Portland.

MATERIALS AND METHODS

Antibodies and Cells—Rabbit polyclonal antibodies against the catalytic domain and against the hinge region of MT1-MMP were from Chemicon (Temecula, CA), Sigma, and Triple Point Biologics (Portland, OR). Rabbit polyclonal antibodies 4b and M8 to the C-terminal and N-terminal parts of pericentrin, respectively, were characterized earlier (24, 25). A murine monoclonal antibody against γ-tubulin was from Sigma. Monoclonal antibodies against α-tubulin, RAB-4 and RAB-11, were from BD Biosciences.

Human U251 glioma, human MCF7 breast carcinoma, and Madin-Darby canine kidney (MDCK) cells were from ATCC (Manassas, VA). All cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. For MT1-MMP overexpression, MDCK cells were transfected with the pcDNA3.1-zeo vector (mock cells) and with the plasmid bearing human MT1-MMP to overexpress the protease. Control and MT1-MMP-expressing breast carcinoma MCF7 and glioma U251 cells were obtained earlier (18, 26). In this work, U251 cells were also transfected with α1-antitrypsin Portland (PDX). MCF7 cells were also transfected with the catalytically inert MT1-MMP-E240A construct and the internalization-deficient, tailless MT1-MMP-ΔCT construct. MCF7 cells were also transfected with MT1-MMP tagged with a FLAG tag. To avoid interference with the trafficking of MT1-MMP, the FLAG tag was inserted into the hinge region of the protease. Peptide cleavage and the mass spectrometry analysis of the digest were performed as described earlier (27). All of the buffer solutions used for the preparation of cell lysates and for the isolation of centrosomes were supplemented with a protease inhibitor mixture (pepstatin, leupeptin, bestatin, aprotinin, E-64) and additionally with phenylmethylsulfonyl fluoride and EDTA (1 mM each).

MT1-MMP Small Interfering (si)RNA Constructs—The MT1-MMP siRNA target sequence was designed by using the siRNA Designer software (Promega). From six tested sequences, the sequence 5'-GAAGCCUGGCUACAGCAAUAU-3' repressed the expression of MT1-MMP most efficiently. The 5'-GGUCCAUGCUGCAGAAAACU-3' scrambled RNA sequence was used as a control in our studies. Both sequences were cloned into the psiLentGene vector (Promega) and used to transfect U251 cells. Transfected cells were selected and cloned in the medium supplemented with 2 μg/ml puromycin. The level of expression of MT1-MMP in the clones was determined by Western blotting.

Isolation of Centrosomes—Centrosomes were isolated from nocodazole-synchronized metaphase U251 cells (25). Mitotic cells were harvested by mitotic shake off and lysed in 1 mM Tris-HCl, pH 8.0, containing 0.5% Igepal. Cell lysates were spun at $1500 \times g$ to separate the nuclei and cell fragments. The supernatant fractions were filtered through a nylon mesh (70-μm pore size) and centrifuged on a 20% w/w Ficoll-400 cushion at 12,000 rpm for 30 min. The crude centrosomal fraction localized at the Ficoll-water interface was collected and further purified by a 40–80% sucrose gradient centrifugation at 30,000 rpm for 2 h.

Immunofluorescence—Cells were fixed in 4% paraformaldehyde for 10 min, permeabilized with 0.1% Triton X-100 for 5 min, and blocked with 1% bovine serum albumin. Cells were incubated with primary antibodies (1:400) for 4 h and then with secondary antibodies (1:200) for 2 h. DNA was stained with 4',6-diamidino-2-phenylindole. Images were acquired at a 600× original magnification on a Nikon TE300 microscope equipped with a real time, cooled CCD camera SP402-115 (Diagnostic Instruments, Sterling Heights, MI).

MMP-2 Activation Assays—The ability of cellular MT1-MMP to activate proMMP-2 was demonstrated by gelatin zymography. For the analysis of centrosomal MT1-MMP, the isolated centrosomes were diluted 1:100 in 25 mM HEPES, pH 7.5. Diluted aliquots were co-incubated for 14 h at 37 °C with the purified proMMP-2 (10 ng). The samples were further analyzed by gelatin zymography.

Fluorescence-acitvated Cell Sorter Analysis—Cells were detached in trypsin-EDTA, fixed in 70% ethanol, washed in phosphate-buffered saline, and resuspended in a 1% bovine serum albumin, phosphate-buffered saline solution supplemented with 50 μg/ml propidium iodide. The DNA content of cells was analyzed on a FACScan flow cytometer.

Metaphase Spreads and Chromosome Count—Cells were incubated for 30 min at 37 °C with 0.005% ethidium bromide and then with colcemid (50 μg/ml) for 2.5 h. Cells were next treated with 0.56% KCl for 15 min and then fixed with Carnoy's fixative. The fixed cells were mounted on glass slides. After 72 h, chromosomes were stained with Giemsa stain and examined on a microscope. Digital images of chromosome spreads were analyzed, and chromosomes were counted in >100 spreads of each cell line.

The Design of the MT1-MMP Chimeras—Using a QuikChange mutagenesis system (Stratagene), the Asp-Tyr-Lys-Asp-Asp-Asp sequence was inserted immediately prior to the Asp^{307}-Lys^{308} sequence of MT1-MMP. As a result, the final construct exhibited the Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys sequence of the FLAG tag in the hinge region of MT1-MMP. To construct MT1-MMP-GFP, the Thr^{300}-Ser^{301} sequence of the hinge domain of MT1-MMP was modified to insert PacI and BlpI restriction sites. The enhanced GFP sequence (Clontech) flanked at both ends with (Gly)$_5$ was then inserted into the PacI/BlpI sites of the MT1-MMP to generate the MT1-MMP-GFP chimera. MCF7 and U251 cells were stably transfected with the pcDNA3.1-zeo plasmids bearing MT1-MMP-FLAG and MT1-MMP-GFP, respectively. To avoid the aberrant trafficking of the recombinant constructs, the clones expressing low levels of the chimeras were specifically selected and analyzed further.

The Analysis of Tumor Biopsies—Frozen samples of colon adenocarcinomas and invasive mammary grade II-III carcinomas and the matched normal tissues were obtained from the NCI Cooperative Human Tissue Network. The homogenized samples were extracted on ice with a radioimmune precipitation assay buffer containing the protease inhibitors. The extract aliquots (60 μg of each) were analyzed by immunoblotting with the MT1-MMP Ab815 and pericentrin 4b antibodies.

RESULTS AND DISCUSSION

Centrosomal MT1-MMP—We examined the subcellular localization of endogenously expressed MT1-MMP in breast carcinoma MCF7 and glioma U251 cells, both of which synthesize MT1-MMP naturally. The level of MT1-MMP in MCF7 cells was, however, very low. U251 cells (Fig. 1*a*) and MCF7 cells (not shown) demonstrated specific centrosomal MT1-MMP immunoreactivity. The centrosomal association of MT1-MMP was confirmed by using γ- and α-tubulin as centrosomal and mitotic spindle markers, respectively. Excess antigen blocked the centrosomal MT1-MMP immunoreactivity (Fig. 1*d*).

Several individual antibodies to MT1-MMP, which were raised against the hinge region and against the catalytic domain, generated similar MT1-MMP immunostaining. The staining of cells with the isotype control was negative. The centrosomal MT1-MMP immunoreactivity was strongly enhanced in the dividing metaphase cells. Overall, only a fraction of MT1-MMP accumulates in centrosomes, whereas the bulk of cellular MT1-MMP is associated with the plasma membrane and the multiple intracellular vesicles (Fig. 1*b*). Nocodazole abrogated the association of MT1-MMP with centrosomes in the interphase cells. Nocodazole had no effect on the association of MT1-MMP with centrosomes in the metaphase cells (Fig. 1*a*).

To corroborate further the presence of endogenous MT1-MMP in centrosomes, U251 cells were stably transfected with the siRNA construct (GAAGCCUGGCUACAGCAAUAU). MT1-MMP silencing by siRNA repressed both the expression of cellular MT1-MMP and its centrosomal immunoreactivity (Figs. 1*a* and 2*c*).

To demonstrate the existence of centrosomal MT1-MMP in transfected cells, we used MT1-MMP chimeras. The use of chimeras allowed us to avoid using MT1-MMP antibodies to confirm the centrosomal localization of the protease. The MT1-MMP-GFP construct was detected via the GFP moiety fluorescence without using antibody staining. The FLAG and the GFP protein sequences were both inserted into the hinge region of MT1-MMP. Following transfection of the cells with the chimeric constructs, MT1-MMP-FLAG and MT1-MMP-GFP were each detected in the centrosomes and co-localized with γ-tubulin in breast carcinoma MCF7 and glioma U251 cells, respectively (Fig. 1*c*). The accumulation of the MT1-MMP chimeras in the pericentrosomal space and the partial co-localization with the centrosomes is a result of MT1-MMP overexpression. Evidently, excess MT1-MMP is incapable of fitting into the tight centrosomal compartment.

To further corroborate the presence of MT1-MMP in the centrosomes, we isolated centrosomes from the synchronized
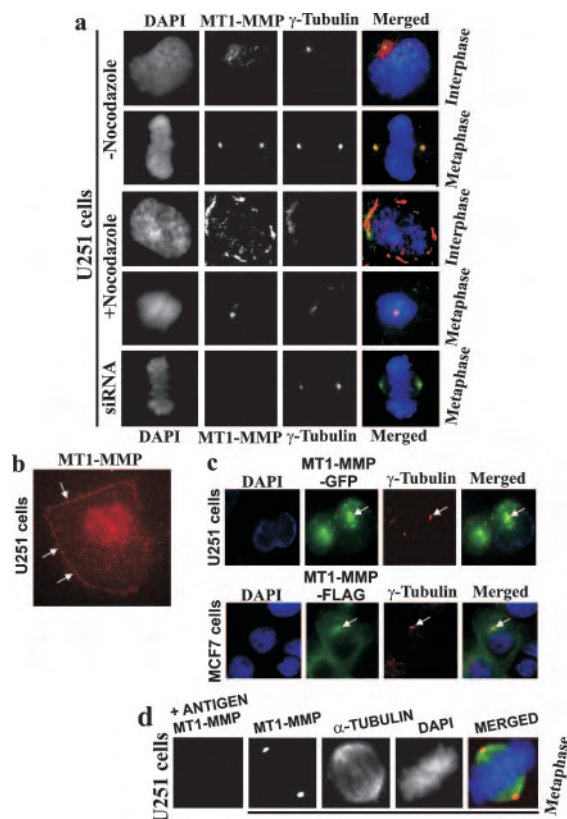
FIG. 1. **Centrosomal MT1-MMP.** *a*, immunostaining of the metaphase and the interphase glioma U251 and breast carcinoma MCF7 cells. Where indicated, cells were pretreated with nocodazole to destroy the cytoskeleton. Silencing by siRNA abrogates MT1-MMP immunoreactivity (in U251 cells, *bottom panel*). An antibody to the catalytic domain of MT1-MMP was used in immunostaining. *b*, immunostaining of endogenously expressed MT1-MMP in U251 cells. *Arrows* point to the plasma membrane. *c*, the MT1-MMP-GFP fluorescent chimera and the MT1-MMP-FLAG chimera in the centrosomes of U251 cells and MCF7 cells, respectively. Anti-FLAG antibody M2 antibody (Sigma) was used to detect the MT1-MMP-FLAG construct. *d*, excess antigen blocks centrosomal MT1-MMP immunoreactivity. The GM6001-inactivated catalytic domain of MT1-MMP (a 10-fold molar excess) was co-incubated with the MT1-MMP antibody for 1 h, and then the sample was used for cell staining (+*antigen MT1-MMP*). The cells were also stained with an untreated MT1-MMP antibody as well as with 4′,6-diamidino-2-phenylindole (*DAPI*) and for an α-tubulin mitotic spindle marker.

metaphase U251 cells and determined that MT1-MMP co-fractionates with γ-tubulin (Fig. 2*a*). The concentration of MT1-MMP in the cytoplasm fraction was significantly lower than that in the centrosomes and that is why the cytoplasm fractions did not demonstrate observable amounts of the protease. In contrast, the centrosome samples were free of MMP-2 (a soluble proteinase and a target of MT1-MMP activation) (Fig. 2*b*) and a plasma membrane marker CD44 (not shown) suggesting the lack of contamination by plasma membrane or transport vesicles.

To demonstrate the functional activity of centrosomal MT1-MMP, purified proMMP-2 was co-incubated with the centrosomal samples. Centrosomal MT1-MMP activated proMMP-2 and converted the latent zymogen proenzyme into the active MMP-2 enzyme (Fig. 2*b*, *bottom panel*). Hydroxamate inhibitors GM6001 and AG3340, which are potent against MT1-MMP ($K_i \approx 0.5$ nm for both inhibitors), blocked MMP-2 activation (not shown). Consistent with the ability of centrosomal MT1-MMP to activate MMP-2, immunoblotting of the purified centrosomes using an MT1-MMP antibody confirmed that centrosomal MT1-MMP is represented by the active enzyme species (Fig. 2*b*, *upper panel*).
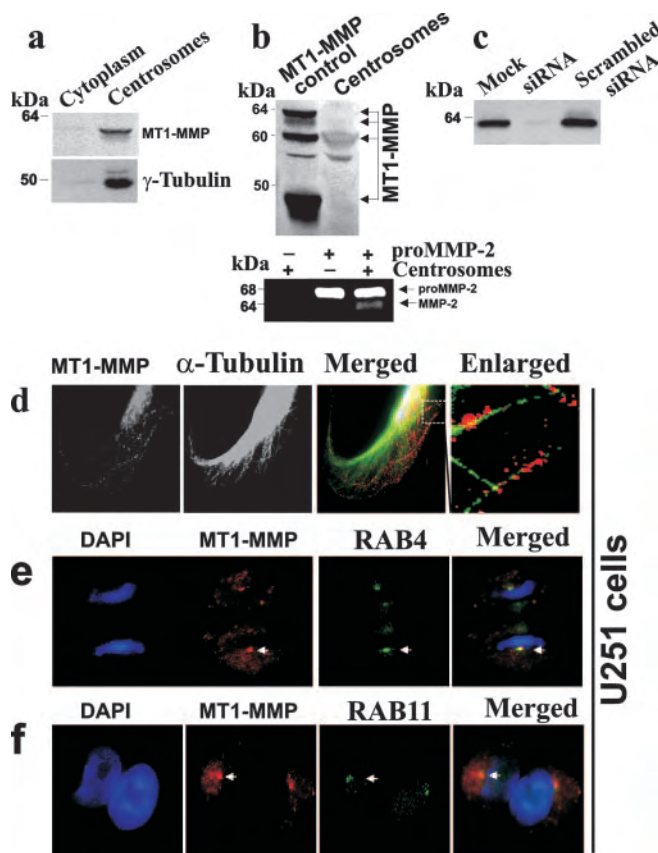
FIG. 2. **Endosomal origin of functionally active centrosomal MT1-MMP.** *a*, immunoblotting confirms co-fractionation of MT1-MMP with centrosomal γ-tubulin in U251 cells. Equal amounts of total protein from the cytoplasm and the centrosomal fractions were analyzed by Western blotting. *b*, gelatin zymography (*bottom panel*) and Western blotting (*upper panel*) demonstrate that centrosomal MT1-MMP is largely represented by the active 60-kDa enzyme and that centrosomal MT1-MMP activates external proMMP-2 and converts the 68-kDa proMMP-2 into the mature 62-kDa MMP-2 enzyme. U251 cells co-expressing MT1-MMP with PDX (a potent inhibitor of furin that is an activator of MT1-MMP) were used as a side-by-side control. PDX/MT1-MMP cells express the proenzyme, the activation intermediate, the mature enzyme, and the 38–45-kDa degraded forms of MT1-MMP. *c*, Western blotting shows that siRNA silencing blocks the expression of cellular MT1-MMP in U251 cells. *d*, MT1-MMP (*red*) is localized alongside the α-tubulin microtubules (*green*) in the interphase cells. *e* and *f*, MT1-MMP (*red*) co-localizes (*arrowheads*) with endosomal markers RAB-4 and RAB-11 (*green*). *DAPI*, 4′,6-diamidino-2-phenylindole.

It is not surprising that MT1-MMP traverses and partially accumulates in the pericentrosomal area, because the microtubule cytoskeleton is essential for the nocodazole-sensitive trafficking of MT1-MMP (28, 29). Centrosomes are the microtubule-organizing centers, which play a key role in rapid protein trafficking. Proteins, *e.g.* caveolin, have been shown to travel from the perinuclear space to the plasma membrane and back using the tubulin cytoskeleton as "railroad tracks" (29, 30).

Our experiments have led us to the discovery that the microtubulin cytoskeleton and the centrosomes (the microtubulin cytoskeleton-organizing centers) are essential for the trafficking and the internalization of MT1-MMP and that MT1-MMP is trafficked to the pericentrosomal space most probably in the endosome-like vehicles. An analysis of the cells showed the existence of MT1-MMP-positive vesicles localized alongside the tubulin cytoskeleton (Fig. 2*d*). RAB-4 and RAB-11 (the markers of late/recycling endosomes and pericentrosomal/recycling endosomes, respectively) (31) co-localize with MT1-MMP, suggesting its endosomal nature (29, 32) (Fig. 2, *e* and *f*).

To examine the intracellular trafficking of MT1-MMP, we

used a newly developed non-covalent protein delivery Chariot reagent (33). This non-covalent reagent allows the delivery of proteins, including antibodies, to the inside of the cell compartment. Following the penetration through the cell membrane, the delivered Chariot-antibody complex dissociated inside the cell compartment and liberated the antibody. The liberated, functional antibody then diffused throughout the cell and interacted with the target protein and, thus, allowed the identification of the subcellular compartment that harbors the target protein. The transduction of cells with the antibodies to MT1-MMP, by using a Chariot reagent, as well as the uptake of the MT1-MMP antibody by cells (29) also confirmed the microtubular transport of vesicular MT1-MMP to the centrosomes (not shown). The most recent publication (34) confirms the endosomal nature and the microtubular intracellular trafficking of metalloproteinases such as MMP-2 and MMP-9. These results provide indirect support for the data presented in our manuscript. Taken together, our data suggest that the tubulin cytoskeleton is involved in the rapid, vesicular MT1-MMP trafficking.

MT1-MMP Targets the Centrosome Proteome—Centrosomes play a central role in the organization of the tubulin cytoskeleton and microtubule nucleation by the γ-tubulin ring complex (24, 35, 36). They regulate the mitotic spindle during cell division and provide sister chromatid disjunction (37). Centrosomal MT1-MMP is proteolytically potent, and therefore, it may attack the centrosomal targets. Knowing the identity of these targets is of great importance to a more complete understanding of the tumorigenic function of MT1-MMP. In our earlier work, we identified the cleavage preferences of MT1-MMP through the proteolysis of protein substrates and the substrate phage libraries (27). We determined that the Pro-X-X- $\downarrow$ -$X_{\text{Hydrophobic}}$ collagen-like cleavage motif is not ideally selective for MT1-MMP because this motif is recognized by several other individual MMPs. Highly selective MT1-MMP substrates lack the characteristic Pro at the P3 position; they contain, instead, an Arg at the P4 position (27). This P4 Arg is essential for efficient hydrolysis and for selectivity for MT1-MMP (38). MT1-MMP appears to recognize cleavage substrates in two distinct modes, using contacts at the P3 and the P1′ to recognize less selective substrates and using contacts at the P4 and the P1′ to recognize highly selective substrates (27).

We used these data to construct a probabilistic cleavage profile of MT1-MMP using a system for the prediction of protease specificity (PoPS) (39). Using a conventional set of parameters such as charge, polarity, and size, the phage library data for the P4–P1′ positions were used to produce a position specific scoring matrix on a scale of -5.0 to $+5.0$, as required by PoPS. The matrix contained a strong preference for Arg at P4 and excluded non-hydrophobic residues from the P1′ position. The matrix was also biased against collagen-like cleavage sites by excluding Pro from the P4 position. Lastly, the matrix was weighted in favor of the P4 and P1′ positions. To filter these predictions further, the programs PSIPRED (40) and NCOILS (41) (integrated in the PoPS system) were used to predict secondary structure and to search for sites that were located in regions of low structure. PoPS was then used to search for the presence of this profile in the human proteome (>25,000 proteins) and in the centrosomal proteome consisting of 114 proteins (42).

This analysis returned a score for each identified site, based on the weighted matrix. The analysis revealed 111 top scoring hits in the human proteome. A significant fraction of known MT1-MMP cleavage targets, including tissue transglutaminase, fibronectin, vitronectin, the low density lipoprotein receptor-related protein LRP, and the complement component C3
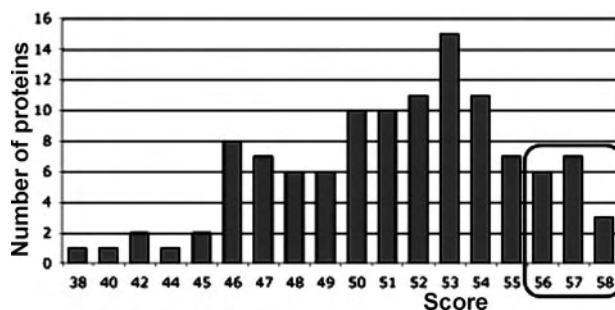
FIG. 3. **PoPS analysis of the centrosomal proteome for the putative cleavage targets of MT1-MMP.** Distribution of the 114 known centrosomal proteins by score is shown. The high scoring centrosomal proteins are *encircled*. Three proteins, including pericentrin, have the highest score of 58.

(43–48) were in this group. The subset of centrosomal proteins was significantly enriched in the high scoring, MT1-MMP-sensitive hits compared with the whole human proteome; ~14% (total of 16) centrosomal proteins have the highest scores of 56–58 (60 is the highest possible score in PoPS), compared with ~2.4% in the same score group of the entire proteome. Of the 111 human top scoring proteins, three proteins are of centrosomal origin.

Fig. 3 shows the number of the known centrosomal proteins that were assigned the "MT1-MMP cleavage score" according to PoPS. One of the three top-scoring targets was the integral centrosomal protein, pericentrin (PoPS score = 58). Two other top-scoring targets were centrosomal Nek-2-associated protein 1 and a protein with an unknown function, KIAA1731. Overall, our *in silico* analyses suggest that centrosomes, relative to the total human proteome, are strongly enriched in the MT1-MMP cleavage targets and that the cleavage of the centrosomal proteins is an important proteolytic function of MT1-MMP.

Pericentrin Is an MT1-MMP Cleavage Target—Pericentrins 1 and 2, which are the splice variants of the same chromosomal gene (GenBank PCN2_HUMAN), are integral and essential centrosomal proteins (49). Pericentrin directly binds γ-tubulin and anchors the γ-tubulin-containing ring complexes to the centrosomes (50). Pericentrin silencing and mutations interfere with normal spindle formation and γ-tubulin localization in the centrosomes and result in G_2 cell-cycle arrest, chromosome instability, and mitotic spindle aberrations (25, 36). The proteolyzed pericentrin was routinely observed in tumor cell lines (24, 25, 36). No individual proteases capable of cleaving pericentrin, however, have been identified so far. Inhibitors of serine and aspartic proteases as well as the specific inhibitors of calpain and caspases and proteasome inhibitors failed to inhibit the proteolysis of cellular pericentrin.

To assess whether pericentrin is susceptible to cleavage by MT1-MMP and to confirm our computer predictions, we synthesized the 10-mer peptides derived from the putative cleavage sites of pericentrin. The peptides were subjected to cleavage by the individual catalytic domain of MT1-MMP at a 1:1000 enzyme:substrate ratio. Mass spectrometry was used to determine the mass of the cleavage products and the localization of the scissile bond (Fig. 4*a*). The A42A peptide (SGAIGF $\downarrow$ LRTA) that is highly sensitive to MT1-MMP (27) was used as a control. GM6001 fully blocked the cleavage of the A42A peptide, thus confirming the absence of contaminating proteases in the MT1-MMP samples. From 12 tested peptides, only the pericentrin peptides bearing the predicted ALRRLLG[1156] $\downarrow$ L[1157]FG and RAARVLG[672] $\downarrow$ L[673]ET cleavage sites were susceptible to MT1-MMP.

We examined further the ability of MT1-MMP to cleave pericentrin in the purified centrosome sample *in vitro*. To avoid
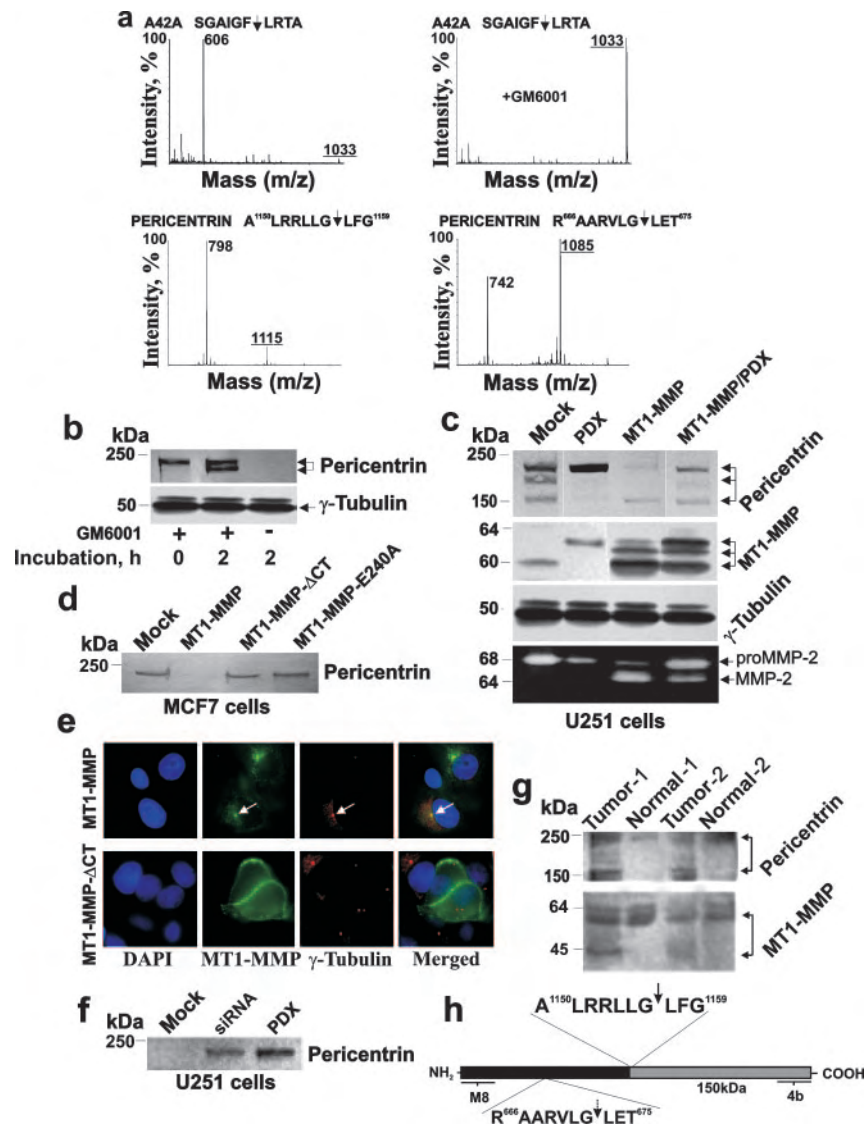
FIG. 4. **MT1-MMP cleaves pericentrin.** *a*, mass spectrometry of the A42A peptide (cleavage control) and the peptides that represent the potential MT1-MMP cleavage sites in pericentrin prior to and after the cleavage by MT1-MMP. The mass of the undigested peptides is *underlined*. Where indicated, GM6001 was added to inactivate MT1-MMP. The cleaved bond is indicated by an *arrow*. The predicted mass of the A[1150]LRRLLG and R[666]AARVLG cleavage products is 797.99 and 741.88 Daltons, respectively. *b*, Western blotting of centrosomal pericentrin and γ-tubulin. The centrosomes were purified from U251 PDX cells. The samples of the purified centrosomes (20 μg of total protein each) were incubated for 2 h with the recombinant catalytic domain of MT1-MMP (200 ng). Where indicated, GM6001 (1 μM) was added to the samples. *c*, immunoblotting (*upper panels*) of centrosomal pericentrin (the 4b antibody against the C-terminal portion of pericentrin was used), cellular MT1-MMP, and γ-tubulin (loading control) from cells transfected with the original plasmid (*mock*), and the plasmids expressing PDX and MT1-MMP alone or in combination (*MT1-MMP/PDX*). Gelatin zymography (*bottom panel*) shows the activation status of proMMP-2, naturally synthesized by the cells. *d*, immunoblotting (the M8 antibody against the N-terminal portion of pericentrin) of cellular pericentrin in total cell lysate of mock MCF7 cells, MCF7 cells expressing the wild type MT1-MMP, the catalytically inert MT1-MMP-E240A, and internalization-deficient, tailless MT1-MMP-ΔCT mutants. *e*, uptake of the MT1-MMP Ab815 antibody by MCF7 cells followed by immunostaining confirms that tailless MT1-MMP-ΔCT (in contrast to the wild type MT1-MMP construct) is not efficiently internalized and, therefore, is incapable of trafficking to the centrosomes and cleaving pericentrin. *Arrows* point to the centrosomes. Antibody uptake by the cells was performed as described earlier (29). *f*, immunoblotting (with the M8 antibody) of cellular pericentrin from total cell lysate demonstrates that both MT1-MMP siRNA silencing and PDX rescue cellular pericentrin in glioma U251 cells. *g*, breast carcinomas exhibit active MT1-MMP and the pericentrin cleavage fragment. Mammary carcinoma biopsies (*tumors 1 and 2*) and matched normal tissue (*normal 1 and 2*) were extracted in the presence of the protease inhibitors. The extracts were analyzed by immunoblotting with the antibodies against MT1-MMP Ab815 and pericentrin 4b. Note that up-regulated pericentrin is cleaved in tumors. *h*, the pattern of pericentrin cleavage and the positions of the pericentrin antibody binding sites. The antibodies M8 and 4b recognize the N-terminal and the C-terminal portions of the pericentrin molecule, respectively. The stable C-terminal 150-kDa fragment frequently accumulates in tumor cells, whereas the N-terminal fragment appears to degrade completely.

the degradation of pericentrin by endogenous MT1-MMP, we purified the centrosomes from U251 cells transfected with α1-anti-trypsin Portland (PDX) (51, 52). In these cells, MT1-MMP is present in the latent proenzyme form, because furin (an activator of MT1-MMP) is repressed by PDX. Co-incubation of the purified centrosomal samples with the recombinant catalytic domain of MT1-MMP followed by the Western blotting of the digest demonstrated the sensitivity of pericentrin to MT1-

MMP. GM6001 rescued pericentrin from MT1-MMP cleavage. Because the antibody M8 to the N-terminal portion of pericentrin was used, the C-terminal cleavage fragments were not observable in this experiment. In turn, γ-tubulin was unaffected by this treatment (Fig. 4*b*). These data argue that centrosomal pericentrin is a likely target of MT1-MMP proteolysis *in vivo*.

To confirm the MT1-MMP cleavage of pericentrin in the cell

system, we analyzed MT1-MMP-transfected and mock-transfected breast carcinoma MCF7 and glioma U251 cells. U251 cells naturally synthesize MMP-2 that can be activated given that MT1-MMP activity is increased in the cells because of transfection with the MT1-MMP cDNA. Mock cells, which were transfected with the empty vector, synthesize MT1-MMP naturally, whereas MT1-MMP-transfected cells overexpress the protease. We also analyzed U251 cells which express the MT1-MMP siRNA or PDX alone or co-express PDX with MT1-MMP. PDX is a potent inhibitor of the proprotein convertases that activate the latent MT1-MMP zymogen (53). As a result, U251 cells, transfected with PDX alone, exhibited only the latent, naturally synthesized zymogen of MT1-MMP and were incapable of activating MMP-2 (Fig. 4c). Cells transfected with MT1-MMP alone exhibited significant levels of the mature MT1-MMP enzyme. In U251 cells, transfected with both MT1-MMP and PDX, the latter significantly albeit incompletely, repressed both the activation of overexpressed MT1-MMP and its ability to activate exogenous proMMP-2. An immunoblotting analysis demonstrated a direct correlation of MT1-MMP activity with the proteolysis of pericentrin (Fig. 4c). In mock glioma cells, which naturally express MT1-MMP, pericentrin was predominantly represented by the intact 220-kDa species (25, 54), and the 200- and 150-kDa degradation fragments. We conclude from these data that the observed limited cleavage of pericentrin is a function of endogenously expressed MT1-MMP rather than MT1-MMP overexpression. In cells overexpressing active MT1-MMP, intact pericentrin disappears, thus confirming the function of MT1-MMP in the cleavage of pericentrin. In turn, the glioma PDX cells, with latent MT1-MMP, exhibit intact pericentrin. The molecular weight of the 150-kDa degradation fragment correlates well with cleavage of pericentrin by MT1-MMP at the ALRRLLG^{1156} ↓ L^{1157}FG site (numbering is given according to pericentrin 2). In these experiments we used the pericentrin antibody 4b that is directed to the C-terminal portion of the protein and that, therefore, recognizes the C-terminal 150-kDa cleavage fragment.

In agreement with the MT1-MMP proteolysis of pericentrin observed in glioma cells, intact pericentrin was not found in MT1-MMP-overexpressing breast carcinoma MCF7 cells (Fig. 4d). To the contrary, the expression of the internalization-deficient, tailless MT1-MMP-ΔCT mutant (Fig. 4e), which is not delivered to the centrosomes, or the catalytically inert MT1-MMP-E240A construct (the Ala substitutes for an essential active site Glu^{240}) rescued pericentrin from the proteolysis in MCF7 cells (Fig. 4d). Similar to PDX, the MT1-MMP siRNA-silencing rescued pericentrin from MT1-MMP cleavage in U251 cells (Fig. 4f).

To confirm our hypothesis that MT1-MMP causes proteolysis of pericentrin, we examined invasive mammary carcinoma, colon adenocarcinoma biopsies, and matching normal tissues. The samples were extracted with a radioimmune precipitation assay buffer containing the protease inhibitor mixture, phenylmethylsulfonyl fluoride and EDTA. MT1-MMP and pericentrin were each assessed by immunoblotting of the extracts. The intact ≈220-kDa pericentrin was found in the normal tissues. In contrast, the 150-kDa degradation fragment of pericentrin was found in mammary carcinoma and colon carcinoma biopsies. In colon carcinoma samples (not shown) the pattern of pericentrin was similar to that observed in breast cancer biopsies (Fig. 4g). The presence of proteolyzed pericentrin in tumor biopsies correlated with the presence of the 45-kDa form of MT1-MMP, which is indicative of MT1-MMP self-proteolysis and, consequently, the protease activity. The pattern of pericentrin cleavage and the positions of the pericentrin antibody binding sites are summarized in Fig. 4h.

Overall, our data suggest that pericentrin is the cleavage target of MT1-MMP *in vivo*. MT1-MMP proteolysis of pericentrin, however, is limited and results in the generation of the 150-kDa degradation fragment, which is associated, as well as intact pericentrin, with the centrosomes. Additional studies are required to identify the function of the pericentrin fragment in malignancy. Consistent with our data, pericentrin also interacts with the cation channel polycystin-2 membrane protein (55), thereby providing evidence of the interactions between membrane and centrosomal proteins. Conversely, interactions of pericentrin with polycystin-2 provide a rationale for the similar interactions of pericentrin with MT1-MMP. The most recent data suggest that a vesicular form of pericentrin also exists in the cells and that vesicular pericentrin could be, in fact, the target of MT1-MMP proteolysis.[2] On the other hand, MT1-MMP is known to autolytically shed its highly potent ectodomain, which could be the major soluble form of intracellular MT1-MMP (56) following the release of the endosomal cargo.

It is highly likely that pericentrin is not a singular intracellular target of MT1-MMP. Our additional proteomics study of the centrosome proteome (~400 individual proteins in glioma U251 cells) demonstrated that ~30 centrosomal proteins represent potential targets of MT1-MMP because they distinguish the cells in which MT1-MMP was silenced by siRNA from the cells in which MT1-MMP was overexpressed. The identification of these putative centrosomal targets of MT1-MMP by mass spectrometry analyses of the tryptic digest fragments is currently in progress.

MT1-MMP Induces Chromosome Instability—To test the hypothesis of whether MT1-MMP causes aberrations in genome inheritance, MDCK epithelial cells were transfected with human MT1-MMP. Tumor cell lines, including U251 and MCF7, demonstrate preexisting chromosome instability and multiple spindle aberrations and, therefore, cannot be used for the identification of MT1-MMP-induced chromatin aberrations. We selected MDCK cells because the conditional expression of human MT1-MMP is, by itself, sufficient to confer tumorigenicity on these non-malignant epithelial cells and to cause the formation of invasive tumors (10). From numerous stably transfected MDCK clones, we selected clones number 5 (MT#5) and number 6 (MT#6) with the high and the low expression of MT1-MMP, respectively, for the analysis (Fig. 5, *a* and *b*). As a control we used MDCK cells transfected with the empty vector (mock). The MT#6 clone demonstrated the centrosomal MT1-MMP immunoreactivity (Fig. 5c). Similar immunoreactivity of MT1-MMP was determined in the MT#5 clone. As expected, pericentrin was strongly degraded in both the MT#5 and MT#6 clones (not shown).

As detected by fluorescence-activated cell sorting, the total DNA content was increased in MT#6 and markedly so in MT#5 cells at 2 months following transfection (Fig. 5d). In contrast, the total DNA content in MDCK cells expressing the tailless, internalization-deficient MT1-MMP-ΔCT construct was close to that in mock cells.

We also identified the number of chromosomes in the cells. There was a direct correlation between the MT1-MMP expression and the DNA content/aneuploidy (Fig. 5, *a*, *b*, and *d*). Mock cells contained 80.2 ± 0.87 chromosomes with a 10% aneuploid frequency. In the MT1-MMP-transfected cells both of these figures were significantly higher (89.1 ± 2.1 chromosomes/27% aneuploidy in MT#6 cells, and 100.3 ± 2.9 chromosomes/48% aneuploidy in MT#5 cells). We inferred that MT1-MMP induced aneuploidy in MDCK cells in a dose-dependent manner.

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[2] S. Doxsey, unpublished observations.

FIG. 5. **Human MT1-MMP induces chromosomal instability in MDCK cells.** *a*, immunoblot of MT1-MMP from mock, MT#5 and MT#6 cells (*upper panel*, the antibodies to the hinge domain were used). The density of the digitized MT1-MMP bands is shown in the *bottom panel*. *b*, chromosome count in mock, MT#5, and MT#6 cells. *c*, immunostaining shows co-localization of human MT1-MMP (*red*) with centrosomal γ-tubulin (*green*) in MT#5 cells. No MT1-MMP immunoreactivity was observed in mock cells. An antibody to the hinge domain of MT1-MMP was used in immunostaining. *d*, fluorescence-activated cell sorter analysis of genomic DNA and the representative chromosomal spread in mock and MT#5 cells. The DNA content in the cells expressing the tailless, internalization-deficient MT1-MMP-ΔCT construct (not shown for the clarity of the figure) was close to that in mock cells. *N*, chromosome number. *e*, immunostaining of mitotic spindle aberrations in MT#5 cells. Chromosomes, α-tubulin, and MT1-MMP are *blue*, *green*, and *red*, respectively.
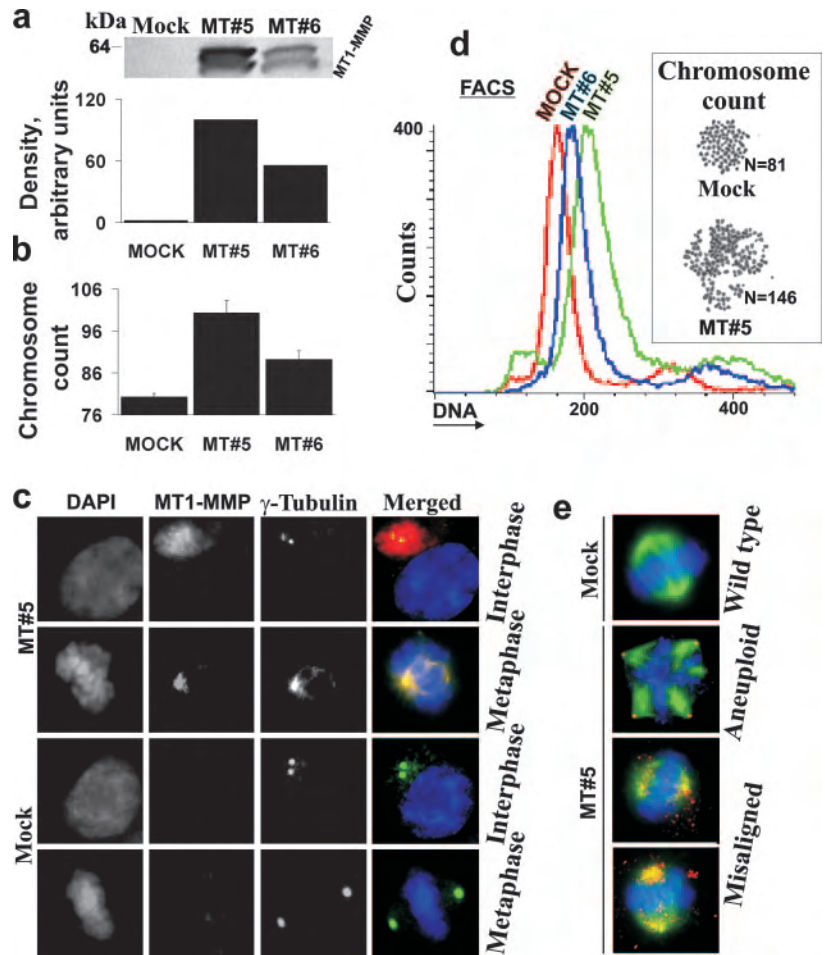
Immunofluorescent staining revealed numerous aberrations of the mitotic spindle in metaphase MT#5 cells (Fig. 5*e*). We concluded, therefore, that MT1-MMP enhances chromosome instability in MDCK cells. These data are consistent with the enhanced tumorigenesis observed in the MT1-MMP-expressing MDCK xenografts in immunodeficient mice (10).

The aberrant functionality of centrosomes correlates with chromosome instability, a predictor of carcinogenesis (57–61). Cells with multiple centrosomes tend to form multipolar spindles, which result in abnormal chromosome segregation during mitosis (57, 62–65). It has been postulated that centrosome aberration may compromise the fidelity of cell division and cause chromosome instability. The acquisition of genomic instability is a crucial step in the development of human cancer (66). The ubiquity of aneuploidy in human cancers, particularly in solid tumors, suggests a fundamental link between errors in chromosome segregation and tumorigenesis. The observed aneuploidy in MT1-MMP-expressing cells suggests the presence of a novel, previously uncharacterized proteolytic pathway to chromatin instability.

It is also highly likely that cellular proteases exhibit the additional, previously unexpected, functions in mitosis. Thus, activation of μ-calpain during mitosis is required for cells to establish the chromosome alignment, suggesting that this protease is also involved in the cleavage of certain centrosomal proteins (67). Consistent with our hypothesis, MMP-2 is present and functions in the nucleus of cardiac myocytes (68). It is premature to extrapolate our data to other members of the MT1-MMP family. We suspect, however, that MT2-MMP and MT3-MMP, similar to MT1-MMP, are likely to be found in the centrosomes and to function in the pericentrosomal compart-ment. It appears also that pericentrin is not a single intracellular target of MT1-MMP. Additional targets of MT1-MMP proteolysis have already been detected, and an effort to determine their identity is currently in progress.

Overall, we suggest that there is a causal link between MT1-MMP, pericentrin proteolysis, and chromosome instability. We also suggest that an intracellular proteolytic function of MT1-MMP is an important element in the transition of cells from normalcy to malignancy and that this novel function elucidates the close association of MT1-MMP with malignant transformation and cancer.

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Centrosomal Pericentrin Is a Direct Cleavage Target of Membrane Type-1 Matrix Metalloproteinase in Humans but Not in Mice

POTENTIAL IMPLICATIONS FOR TUMORIGENESIS*

Vladislav S. Golubkov[‡], **Alexei V. Chekanov**[‡], **Stephen J. Doxsey**[§], **and Alex Y. Strongin**[‡1]

From the ‡*Cancer Research Center, The Burnham Institute for Medical Research, La Jolla, California 92037 and the* §*University of Massachusetts Medical School, Worcester, Massachusetts 01605*

Membrane type-1 matrix metalloproteinase (MT1-MMP) exhibits its distinctive and important pericellular cleavage functions. Recently, we determined that MT1-MMP was trafficked to the centrosomes in the course of endocytosis. Our data suggested that the functionally important, integral, centrosomal protein, pericentrin-2, was a cleavage target of MT1-MMP in human and in canine cells and that the sequence of the cleavage sites were ALRRLLG^{1156} ↓ L^{1157}FG and ALRRLLS^{2068} ↓ L^{2069}FG, respectively. The presence of Asp-948 at the P1 position inactivated the corresponding site (ALRRLLD^{948}-L^{949}FGD) in murine pericentrin. To confirm that MT1-MMP itself cleaves pericentrin directly, rather than indirectly, we analyzed the cleavage of the peptides that span the MT1-MMP cleavage site. In addition, we analyzed glioma U251 cells, which co-expressed MT1-MMP with the wild type murine pericentrin and the D948G mutant. We determined that the D948G mutant that exhibited the cleavage sequence of human pericentrin was sensitive to MT1-MMP, whereas unmodified murine pericentrin was resistant to proteolysis. Taken together, our results confirm that MT1-MMP cleaves pericentrin-2 in humans but not in mice and that mouse models of cancer probably cannot be used to critically examine MT1-MMP functionality.

MT1-MMP^2/MMP-14 is a prototypic member of the membrane-tethered matrix metalloproteinases (1). Although MT1-MMP is present in normal tissues, its enhanced expression is directly linked to tumor progression and metastasis (2–6). Cell surface-associated MT1-MMP is a multifunctional enzyme (7), and it is involved in the pericellular proteolysis of the extracellular matrix, the activation of soluble MMPs, and the cleavage of adhesion and signaling cell receptors (8–10). The functional activity of MT1-MMP is regulated by its activation by furin-like proprotein convertases, by its inhibition by TIMPs, and by its self-proteolysis and shedding (11–13). Evidence is also emerging that exocytosis, endocytosis, and recycling also regulate the presentation of MT1-MMP on the cell surface and, consequently, its cell surface-associated proteolytic activity (14–17).

Recently, we determined that functionally active MT1-MMP, which was presented on the cell surface, was internalized, trafficked alongside the microtubular cytoskeleton, and delivered to the centrosomal compartment (16, 18). The presence of MT1-MMP in the pericentrosomal space correlated with the cleavage of human pericentrin-2 (kendrin), an integral and functionally important centrosomal, 3336-amino-acid residue long, protein (19–22), and chromosome instability in non-malignant epithelial Madin-Darby canine kidney cells (18, 23). Centrosomes, spindle pole bodies, are cellular organelles that exhibit an ability to organize microtubules and to nucleate (24). The normal functionality of centrosomes is essential to the organization of the cytoskeleton and the mitotic spindle, self-duplication, and cell cycle progression (25, 26). Conversely, centrosomal abnormalities, early predictors of carcinogenesis, promote mitotic spindle aberrations and chromosome instability, events which are frequently observed in neoplastic cells (27–31). It is well established that pericentrin supports the normal functioning of the centrosomes and the cytoskeleton and that its function is important to cell cycle progression (27, 32, 33). Despite the evident functional link of MT1-MMP activity with the cleavage of pericentrin observed in both human and canine cells (18), there were suspicions that MT1-MMP is indirectly, rather than directly, involved in these unorthodox, intracellular, proteolytic events. To demonstrate that MT1-MMP cleaves pericentrin directly, we used mutagenesis of murine pericentrin. The peptide sequence of murine, canine, and human pericentrin-2 is homologous. There is, however, a single amino acid substitution at the P1 position of the MT1-MMP cleavage site in murine pericentrin when compared with that of human and canine proteins. Consistent with the cleavage preferences of MT1-MMP (34), we hypothesized that Asp-948 inactivates the cleavage site in murine pericentrin.

Here, we reconstructed the MT1-MMP human cleavage site in the murine pericentrin-2 sequence. Consistent with the proteolysis of human pericentrin-2 at the ALRRLLG^{1156} ↓ L^{1157}FG site, a single D948G mutation transformed murine pericentrin into the cleavage target of MT1-MMP. We suggest that these results confirm that MT1-MMP cleaves pericentrin in humans, but not in mice, and that the intracellular function of centrosomal MT1-MMP in humans cannot be fully recapitulated in the cellular and animal models in mice.

MATERIALS AND METHODS

Reagents—Rabbit polyclonal antibody AB815 to the hinge region of MT1-MMP was from Chemicon (Temecula, CA). Murine monoclonal antibody 5D1 to the MT1-MMP catalytic domain was generated jointly by our laboratory and Chemicon. Rabbit polyclonal antibodies 4b and M8 to the C-terminal and N-terminal parts of pericentrin, respectively, were characterized earlier (22, 35).

The recombinant catalytic domain of human and murine MT1-MMP was each expressed in *Escherichia coli* and then purified from the inclusion bodies and refolded to restore the catalytic activity (36). The pep-

[1] To whom correspondence should be addressed: The Burnham Institute, 10901 North Torrey Pines Rd., La Jolla, CA. Tel.: 858-713-6271; Fax: 858-713-9925; E-mail: strongin@burnham.org.

[2] The abbreviations used are: MT1-MMP, membrane type-1 matrix metalloproteinase; siRNA, small interfering RNA.

MT1-MMP Cleaves Centrosomal Pericentrin

FIGURE 1. **The cleavage of pericentrin-derived peptides by MT1-MMP.** The cleavage control peptide SGRIGF ↓ LRTA and the pericentrin peptides that span the putative MT1-MMP cleavage site, were each cleaved by MT1-MMP. The intact and digest samples were analyzed by mass spectrometry. The mass of intact peptide is *underlined*. The sequence alignment of the putative MT1-MMP cleavage site in human, canine, and murine pericentrin-2 (GenBank™ Accession Numbers O95617, XP_548735, and P48725, respectively) is presented in the upper part of the figure. *Arrows* indicates the scissile bonds.
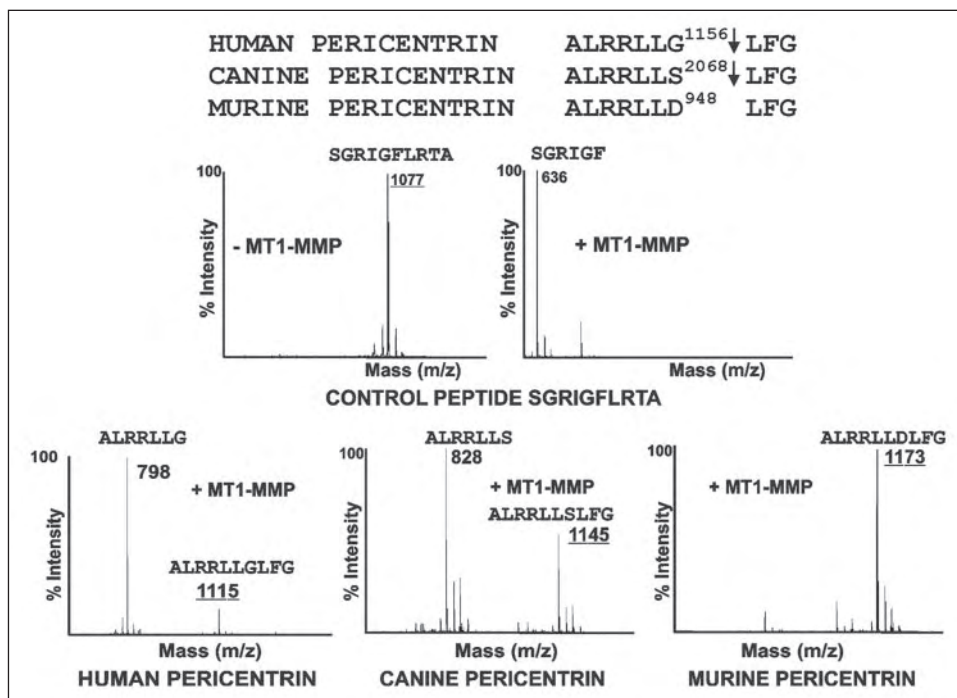
tides ALRRLLGLFG, ALRRLLSLFG, and ALRRLLGLFG, which span the MT1-MMP putative cleavage site in murine, canine, and human pericentrin, respectively, were synthesized by GenScript (Piscataway, NJ). The peptides were cleaved for 2 h by the catalytic domain of MT1-MMP at the enzyme-substrate ratio of 1:1000 and the digest samples were analyzed by MALDI-TOF mass-spectrometry (18, 34).

Mutagenesis and Cell Transfection—The cDNA construct of murine pericentrin was inserted in the pLPX7-blasticidin vector using routine manipulations. The oligonucleotide direct and reverse primers (5′-CTCCGAGATGCCCTGAGGAGACTTCTAGGCCTGTTTGGG-GACACACTGAAGGCAGC-3′ and GCTGCCTTCAGTGTGTCCCC-AAACAGGCCTAGAAGTCTCCTCAGGGCACTCGGAG-3′, respectively; mutant positions are underlined) were used in PCR mutagenesis to insert the D948G mutation in the sequence of murine pericentrin. The presence of the mutation in the mutant construct was confirmed by DNA sequencing.

Mock-transfected human U251 glioma cells (mock), the cells stably overexpressing MT1-MMP (MT cells) and the cells in which MT1-MMP was stably silenced by the 5′-GAAGCCUGGCUACAGCAAUAU-3′ siRNA construct (siMT cells) were constructed and partially characterized earlier (18, 37, 38). The 5′-GGUCCAUGCUGCAGAAAAACU-3′ scrambled siRNA construct was used as a control. Both siRNA constructs were cloned in the psiLentGene-puromycin vector (Promega, Madison, WI). There was no effect of the scrambled siRNA construct on the expression of MT1-MMP in U251 cells (not shown). Cells were routinely grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum.

In this work, mock, MT, and siMT cells were additionally transiently transfected with the pLPX7 plasmid coding for the wild type and the D948G mutant murine pericentrin-2. Blasticidin-resistant cells were selected in 7 days. The expression of murine pericentrin was analyzed by Western blotting of the cell lysates, which were prepared from the total pool of blasticidin-resistant cells. To identify both the N-terminal and the C-terminal fragments of pericentrin, a mixture of 4b and M8 antibodies was used in these experiments. The expression of the murine pericentrin constructs was also analyzed by immunostaining the transfected cells.

Immunofluorescence—Cells were fixed in 4% paraformaldehyde for 10 min, permeabilized with 0.1% Triton X-100 for 5 min, and blocked with 1% bovine serum albumin. Cells were then incubated for 4 h with the primary antibody followed by incubation for 2 h with the species-specific secondary antibody conjugated with green Alexa Fluor 488 or red Alexa Fluor 594 (Molecular Probes, Eugene, OR). DNA was stained with 4′,6-diamidino-2-phenylindole. Images were acquired at a 600× original magnification on a Olympus BX51 fluorescent microscope equipped with a cooled MagnaFire camera (Olympus, San Diego, CA).

General Methods—Gelatin zymography of MMP-2 from medium aliquots, cell surface biotinylation, cell lysate preparation, immunocapture of biotin-labeled MT1-MMP, and Western blotting analysis of MT1-MMP and pericentrin were performed as described in our earlier publications (18, 39). The buffers used for the preparation of cell lysates were supplemented with a protease inhibitor mixture (pepstatin, leupeptin, bestatin, aprotinin, and E-64) and in addition, with phenylmethylsulfonyl fluoride and EDTA (1 mM each) to prevent additional, artificial proteolysis of the lysate samples.

RESULTS

Analysis of the Peptides That Span the MT1-MMP Cleavage Site in Human, Canine, and Murine Pericentrin—Our earlier data suggested that cellular MT1-MMP cleaved human centrosomal pericentrin at the ALRRLLG^{1156} ↓ L^{1157}FG cleavage site (18). Sequence alignment of human, murine, and canine pericentrin shows that the ALRRLLG^{1156} ↓ L^{1157}FG and ALRRLLS^{2068} ↓ L^{2069}FG sequences are present in humans and canines, respectively, whereas mice exhibit the ALRRLL D^{948}L^{949}FG sequence (Fig. 1). In agreement, in the cleavage tests *in vitro*, MT1-MMP cleaved the synthetic peptides that spun the human and canine cleavage site sequences. In contrast, the peptide ALRRLLD^{948}L^{949}FG that corresponded to the sequence of murine pericentrin was resistant to proteolysis by human MT1-MMP. Murine MT1-MMP also did not cleave this peptide (not shown). These data are consistent with the cleavage preferences of MT1-MMP, and they suggest that the presence of a negatively charged Asp residue at the P1

FIGURE 2. **Mutant pericentrin D948G is cleaved by cellular MT1-MMP in glioma U251 cells.** Murine pericentrin-2 and its D948G mutant were each transfected into mock cells (*MOCK/PER-MUR* and *MOCK/PER-D948G*, respectively) and co-expressed with MT1-MMP (*MT/PER-MUR* and *MT/PER-D948G*, respectively). In addition, murine pericentrin and its D948G mutant were co-expressed with the siRNA construct, which silenced the expression of MT1-MMP (*siMT/PER-MUR* and *siMT/PER-D948G*, respectively). For the analysis of MT1-MMP, cells were surface labeled with biotin, biotin-labeled proteins were immunocaptured on streptavidin-beads, and the precipitated samples were analyzed by Western blotting with the MT1-MMP antibody AB815 (*middle panel*). Pericentrin was analyzed by Western blotting of the cell lysates. The mixture of the 4b and M8 antibodies was used to detect pericentrin (*upper panel*). The MT1-MMP-dependent activation of MMP-2 was determined by subjecting medium aliquots to gelatin zymography (*bottom panel*). For gelatin zymography analyses, cells were grown in serum-free medium. Positions of the molecular weight markers are on the left. The pericentrin sequence is shown above the panels.
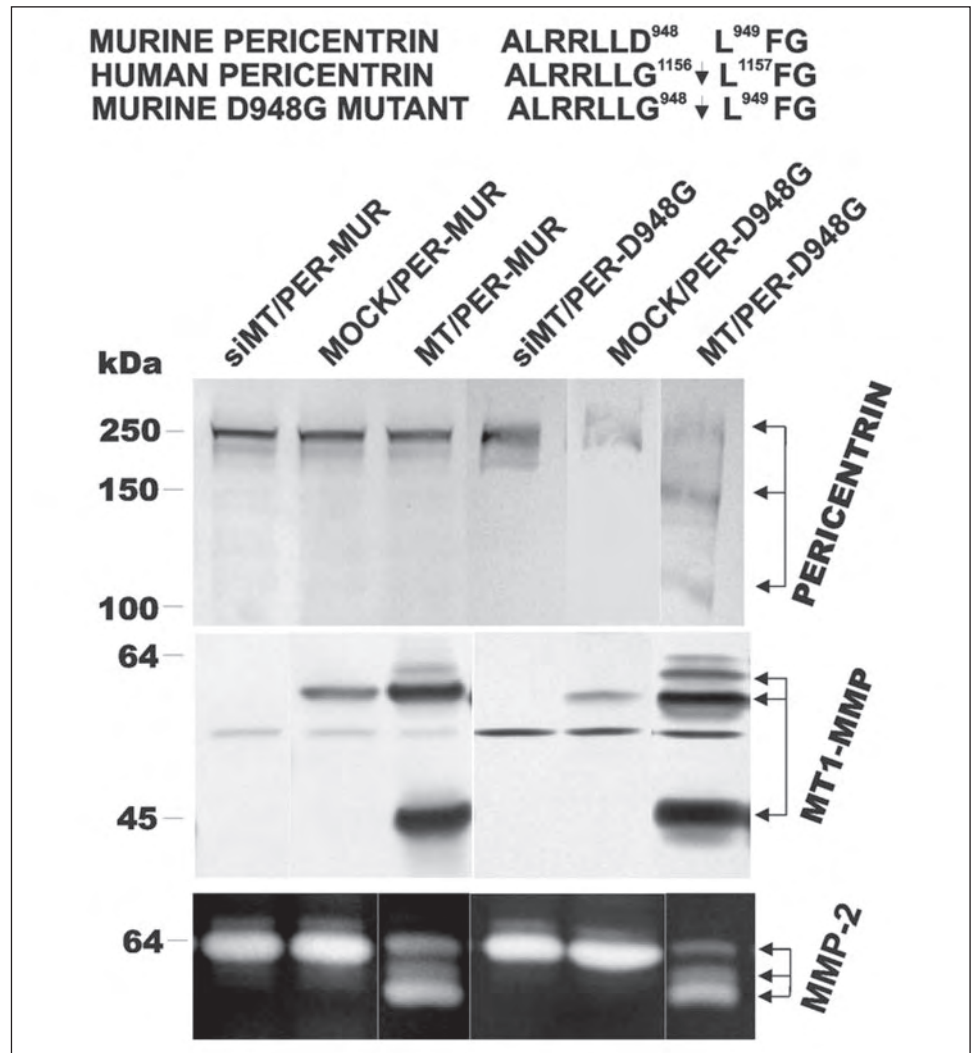
position is likely to inactivate the site and to protect the peptide substrate from MT1-MMP proteolysis (34).

MT1-MMP Cleaves the Murine Pericentrin D948G Mutant That Exhibits the MT1-MMP Cleavage Site—To validate our *in vitro* cleavage data in a cell setting, we first mutated the sequence of murine pericentrin ALRRLLD^{948}L^{949}FG and then isolated the D948G mutant. This mutant exhibited the human MT1-MMP cleavage site ALRRLLG^{948} ↓ L^{949}FG. We specifically selected robust U251 cells for these studies because they, as in other aggressive malignant cells, exhibit the required compensatory mechanisms to overcome the overexpression of pericentrin. Other cell types usually do not survive the overexpression of this multifunctional, integral, centrosomal protein, the levels of which control cell cycle progression and genetic stability (29). We next transfected mock, MT, and siMT cells with the wild type and D948G mutant murine pericentrin. Finally, we determined whether the pericentrin constructs were cleaved in the transfected cells. Fig. 2 shows that the expression of MT1-MMP was silenced in siMT cells. Low levels of MT1-MMP were observed in mock cells, which synthesized MT1-MMP naturally. The expression of MT1-MMP was up-regulated in MT cells, in which a characteristic 42–45-kDa self-degradation form of MT1-MMP was observed in addition to the 60–64-kDa full-length forms of the protease. According to our earlier data and the observations of the others, the presence of the 42–45 self-proteolytic form in

the cell samples indicates the presence of high levels of the catalytically potent MT1-MMP (11, 12).

Both the wild type and the D948G mutant murine pericentrins were stable in mock and siMT cells. The cleavage of the wild type murine pericentrin was not observed in MT cells, which overexpress the protease. In contrast, there was an extensive proteolysis of the D948G mutant in MT cells. The combined size of the observed N-terminal and C-terminal cleavage fragments of pericentrin (105 and 140 kDa, respectively) correlated well with the size of intact murine pericentrin (240–250 kDa). Because these experiments were performed with the individual total cell pools obtained *via* transiently transfecting cells with the pericentrin constructs, the levels of pericentrin differ insignificantly among the samples.

As shown by gelatin zymography of the medium samples, mock and siMT cells did not activate secretory MMP-2, which was produced naturally by glioma cells. Consistent with many other reports (12), transfection of the cells with MT1-MMP stimulated extensive activation of the MMP-2 zymogen and its conversion into the mature enzyme. The efficiency of MMP-2 activation in MT cells co-transfected with either unmodified murine pericentrin or the D948G mutant was similar when compared with the cells expressing MT1-MMP alone (Fig. 2). These results indicated that the intracellular, pericentrin-cleaving function of MT1-MMP and the status of pericentrin do not affect the proteolytic

MT1-MMP Cleaves Centrosomal Pericentrin
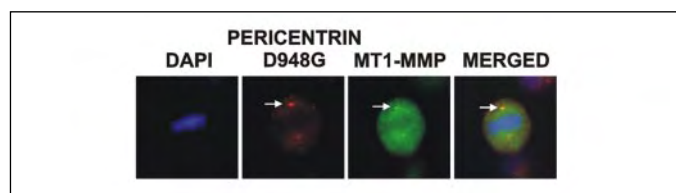
FIGURE 3. **Pericentrin D948 mutant co-localizes with MT1-MMP in the centrosomes.** Pericentrin and MT1-MMP were stained with the antibodies M8 and 5D1, respectively, in permeabilized MT/PER-D948G U251 cells. Nuclei were counterstained with 4′,6-diamidino-2-phenylindole (*DAPI*). *Arrows* indicate the centrosomes.

pericellular function of MT1-MMP. Overall, these results confirmed our *in vitro* peptide cleavage data and suggested that the reconstruction of the MT1-MMP human cleavage site ALRRLLG^{948} ↓ L^{949}FG transformed murine pericentrin into the cleavage target of MT1-MMP. In agreement with the proteolysis of mutant pericentrin by MT1-MMP, there was an evident centrosomal co-localization of these two proteins in the transfected cells (Fig. 3).

DISCUSSION

From the 24 known human MMPs, an elevated expression of MT1-MMP is most closely associated with malignancies (5, 40). There is extensive evidence that cell surface-associated MT1-MMP functions as one of the key players of pericellular proteolysis in humans and mice (3, 7, 8). Knock-out mice models generated volumes of highly valuable information about the proteolytic function of cellular MT1-MMP (41–45). There is evidence, however, that after being delivered to the plasma membrane, MT1-MMP, along with many other membrane-tethered proteins (46, 47), is internalized. The clathrin- and caveolin-dependent internalization pathways are both involved in the internalization and the recycling of MT1-MMP (14, 15). In the pathway of MT1-MMP through the cell compartment, the proteolytically potent MT1-MMP accumulates in the microtubulin cytoskeleton-organizing centers, the centrosomes. Our earlier work (18) suggested that both human and canine pericentrins were cleavage targets of centrosomal MT1-MMP. Pericentrin is an integral centrosomal protein, and it is essential to the normal functioning of centrosomes and to mitotic spindle formation (48). The expression of MT1-MMP in the centrosomes of either human or canine cells correlated with the presence of the proteolytic fragments of pericentrin. In addition, these events correlated with the induction of mitotic spindle aberrations and aneuploidy in non-malignant MDCK cells (18, 23). The sequence alignment of the putative cleavage site in human and canine pericentrin (ALRRLLG^{1156} ↓ L^{1157}FG and ALRRLLS^{2068} ↓ L^{2069}FG, respectively) supported our biochemical and cellular experiments, because both Gly and Ser are compatible with the known cleavage preferences of MT1-MMP. This alignment also suggested that the corresponding sequence region of murine pericentrin (ALRRLLD^{948} ↓ L^{949}FGD) is protected from MT1-MMP proteolysis because of the Asp-948 at the P1 position (underlined in the peptide) in the murine sequence. Consistent with this hypothesis, murine pericentrin was resistant to MT1-MMP. Based on this knowledge, we reconstructed the human cleavage site in the murine pericentrin sequence. As expected, the D948G mutant that exhibited the human cleavage site was cleaved by MT1-MMP. We believe that our experiments proved that MT1-MMP cleaves pericentrin directly, and we suggest that centrosomal MT1-MMP, through the cleavage of pericentrin, plays a unique role in human cells. In addition, the presence of the proteolytic fragments of pericentrin in the tumor biopsies, which express high levels of MT1-MMP, supports an important role of the MT1-MMP/pericentrin axis in cancer (18). Our data imply the pericellular function of MT1-MMP appears to be common across the species, whereas the intracellular,

pericentrin-cleaving function of MT1-MMP is absent in mice. These hypotheses add another level of complexity to be overcome in our attempts to understand completely the tumorigenic functions of MT1-MMP in humans, and they warrant additional studies of the genetically redesigned animal models, so that they will fully recapitulate human tumorigenesis.

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Centriolin Anchoring of Exocyst and SNARE Complexes at the Midbody Is Required for Secretory-Vesicle-Mediated Abscission

Adam Gromley,[1,3] Charles Yeaman,[2] Jack Rosa,[1]
Sambra Redick,[1] Chun-Ting Chen,[1]
Stephanie Mirabelle,[1] Minakshi Guha,[1]
James Sillibourne,[1] and Stephen J. Doxsey[1,*]
[1]Program in Molecular Medicine
University of Massachusetts Medical Center
Worcester, Massachusetts 01605
[2]Department of Anatomy and Cell Biology
University of Iowa
Iowa City, Iowa 52242

Summary

The terminal step in cytokinesis, called abscission, requires resolution of the membrane connection between two prospective daughter cells. Our previous studies demonstrated that the coiled-coil protein centriolin localized to the midbody during cytokinesis and was required for abscission. Here we show that centriolin interacts with proteins of vesicle-targeting exocyst complexes and vesicle-fusion SNARE complexes. These complexes require centriolin for localization to a unique midbody-ring structure, and disruption of either complex inhibits abscission. Exocyst disruption induces accumulation of v-SNARE-containing vesicles at the midbody ring. In control cells, these v-SNARE vesicles colocalize with a GFP-tagged secreted polypeptide. The vesicles move to the midbody ring asymmetrically from one prospective daughter cell; the GFP signal is rapidly lost, suggesting membrane fusion; and subsequently the cell cleaves at the site of vesicle delivery/fusion. We propose that centriolin anchors protein complexes required for vesicle targeting and fusion and integrates membrane-vesicle fusion with abscission.

Introduction

Cytokinesis is a fundamental process that results in division of a single cell with replicated DNA into two daughters with identical genomic composition (see (Glotzer, 2001, 2005; Guertin et al., 2002). Early events in animal cell cytokinesis include assembly and contraction of the actomyosin ring to form the cleavage furrow. Continued furrowing results in constriction of the plasma membrane to form a narrow cytoplasmic bridge between the two nascent daughter cells. Within this intercellular bridge are bundled microtubules and a multitude of proteins that together form the midbody. In a poorly understood final step called abscission, the

cell cleaves at the intercellular bridge to form two daughter cells.

Membrane trafficking is required for late stages of cytokinesis (Albertson et al., 2005; Finger and White, 2002; Jurgens, 2005; Papoulas et al., 2004; Strickland and Burgess, 2004). In *C. elegans* embryos, inhibition of Golgi secretion by brefeldin A (BFA) resulted in late-stage cytokinesis defects (Skop et al., 2001). More recent studies in mammalian cells using dominant-negative approaches showed that the membrane-fusion-inducing SNARE components, syntaxin-2 and endobrevin/VAMP8, are required for a final step in cell cleavage (Low et al., 2003). Endocytic traffic also plays a role in cytokinesis. Recycling endosomes and associated components localize to the midbody and are required for cell cleavage (Monzo et al., 2005; Wilson et al., 2005; Thompson et al., 2002). However, little is known about the spatial and temporal control of dynamic membrane compartments and molecules during abscission or how these activities are coordinated to achieve cell cleavage.

The role of membrane-vesicle-tethering exocyst complexes in animal cell abscission is poorly understood. The exocyst is a multiprotein complex that targets secretory vesicles to distinct sites on the plasma membrane. In the budding yeast *S. cerevisiae*, exocyst components localize to the mother-bud neck, the site of cytokinesis (Finger et al., 1998; Mondesert et al., 1997). Exocyst disruption results in accumulation of vesicles at this site (Salminen and Novick, 1989) and impairs actomyosin-ring contraction and cell cleavage (Dobbelaere and Barral, 2004; Verplank and Li, 2005). In the fission yeast *S. pombe*, exocyst components localize to the actomyosin ring (Wang et al., 2002). Mutants for the exocyst component Sec8 accumulate 100 nm "presumptive" secretory vesicles near the division septum and cannot complete extracellular separation of the two daughter cells. A screen for cytokinesis mutants in *Drosophila melanogaster* identified the exocyst component sec5 (Echard et al., 2004), and proteomic analysis of the midbody in mammalian cells showed that the exocyst protein sec3 is at the midbody (Skop et al., 2004). Mammalian exocyst components are involved in secretion in polarized epithelial cells (Yeaman et al., 2004) and localize to the midbody (Skop et al., 2004; Wilson et al., 2005), but the function of the exocyst during cytokinesis is unclear.

Components of membrane-vesicle-tethering and -fusion complexes have been identified in some organisms and linked to cytokinesis, but the pathway that integrates these complexes with vesicle trafficking during cell cleavage is unknown. Little is known about how SNAREs and the exocyst are anchored at the midbody or how they modulate membrane-vesicle organization and fusion to coordinate abscission. Moreover, the origin and dynamics of membrane compartments involved in abscission have not been investigated. In this manuscript, we describe a multistep pathway for abscission that requires a scaffold protein to anchor
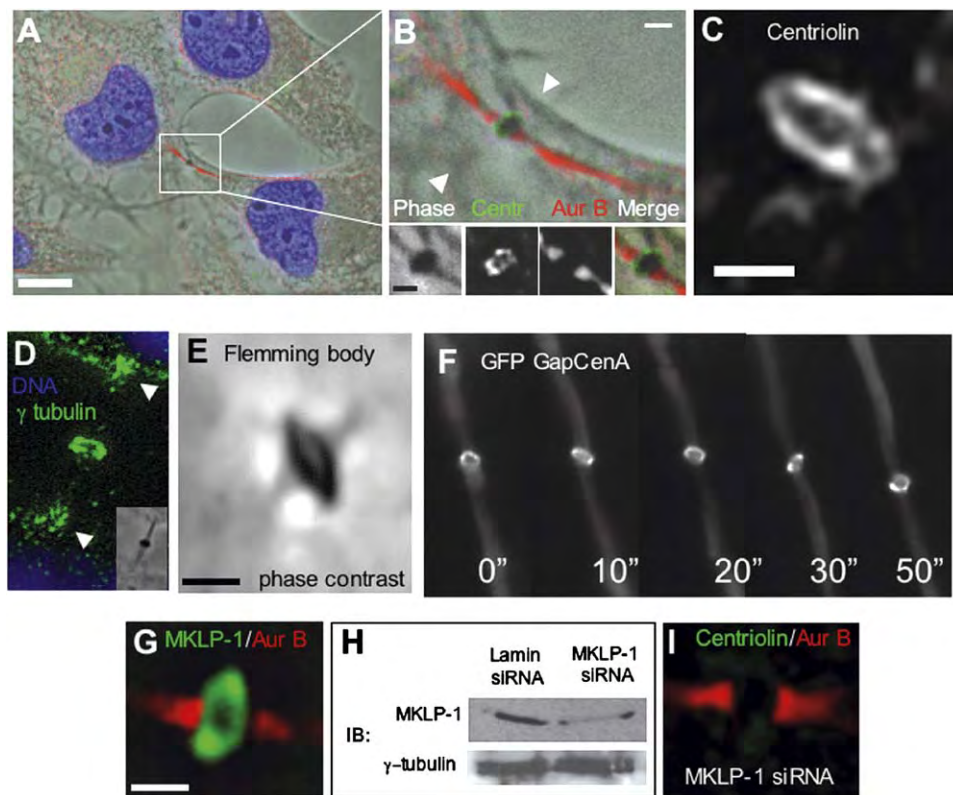
Figure 1. Centriolin Localizes to a Midbody Ring

(A) Immunofluorescence/phase image of HeLa cell during cytokinesis showing the phase-dense Flemming body within the larger diameter of the plasma membrane (arrowheads in [B]).

(B and C). Boxed region enlarged with insets (B) to show the centriolin ring (Centr, enlarged in [C]) as part of the Flemming body (phase) and flanked bilaterally by Aurora B (Aur B).

(D) γ-tubulin localizes to the midbody ring (inset, Flemming body) and sites of presumed microtubule minus ends (arrowheads).

(E) The Flemming body forms a ring.

(F) GFP-tagged GAPCenA localizes to the midbody ring and is highly dynamic (time in s).

(G–I) MKLP-1 localizes to the midbody ring (G) and, upon depletion, mislocalizes centriolin from the midbody (I). Immunoblots (IB) from cells treated with siRNAs targeting MKLP-1 or lamin A/C (control) (H). γ-tubulin, loading control. Scale bars in (A), 10 μm; (B), 5 μm; (C), (E), and (G), 1 μm.

SNARE and exocyst complexes at a unique midbody site and also requires asymmetric transport and fusion of secretory vesicles at this site.

Results

Centriolin Is Part of a Ring-like Structure at the Central Midbody during Cytokinesis

We previously showed that centriolin localized to the midbody during cytokinesis (Gromley et al., 2003). Using high-resolution deconvolution microscopy, we now demonstrate that centriolin is part of a unique ring-like structure within the central portion of the midbody, which we call the midbody ring (observed in ∼75% of all telophase cells, Figures 1A–1C). The midbody ring was 1.5–2 μm in diameter (Figure 1C), contained γ-tubulin (Figure 1D), and colocalized with the phase-dense Flemming body (Figure 1B, inset) (Paweletz, 1967). In fact, high-magnification phase-contrast imaging revealed that the Flemming body was organized into a ring-like structure (Figure 1E). The midbody ring was

flanked by Aurora B kinase, which colocalized with microtubules on either side of the ring (Figure 1B, inset). Several other proteins localized to the midbody ring including ectopically expressed GFP-GAPCenA, a GTPase-activating protein previously shown to localize to centrosomes (Cuif et al., 1999). Time-lapse imaging of GFP-GAPCenA and other proteins in living cells showed that the midbody ring was dynamic, moving between cells and tipping from side to side to reveal the ring structure (Figure 1F; see also Movie S1 in the Supplemental Data available with this article online). In addition, midbody-ring localization of GFP-GAPCenA confirmed the ring structure seen by immunofluorescence microscopy and demonstrated that there were no antibody penetration problems in this midbody region as seen for other antigens (Saxton and McIntosh, 1987). The midbody ring was distinct from the actomyosin ring and did not change in diameter during cytokinesis (Figures 1A and 1B). It appeared during the early stages of actomyosin-ring constriction and persisted until after cell cleavage (see below).
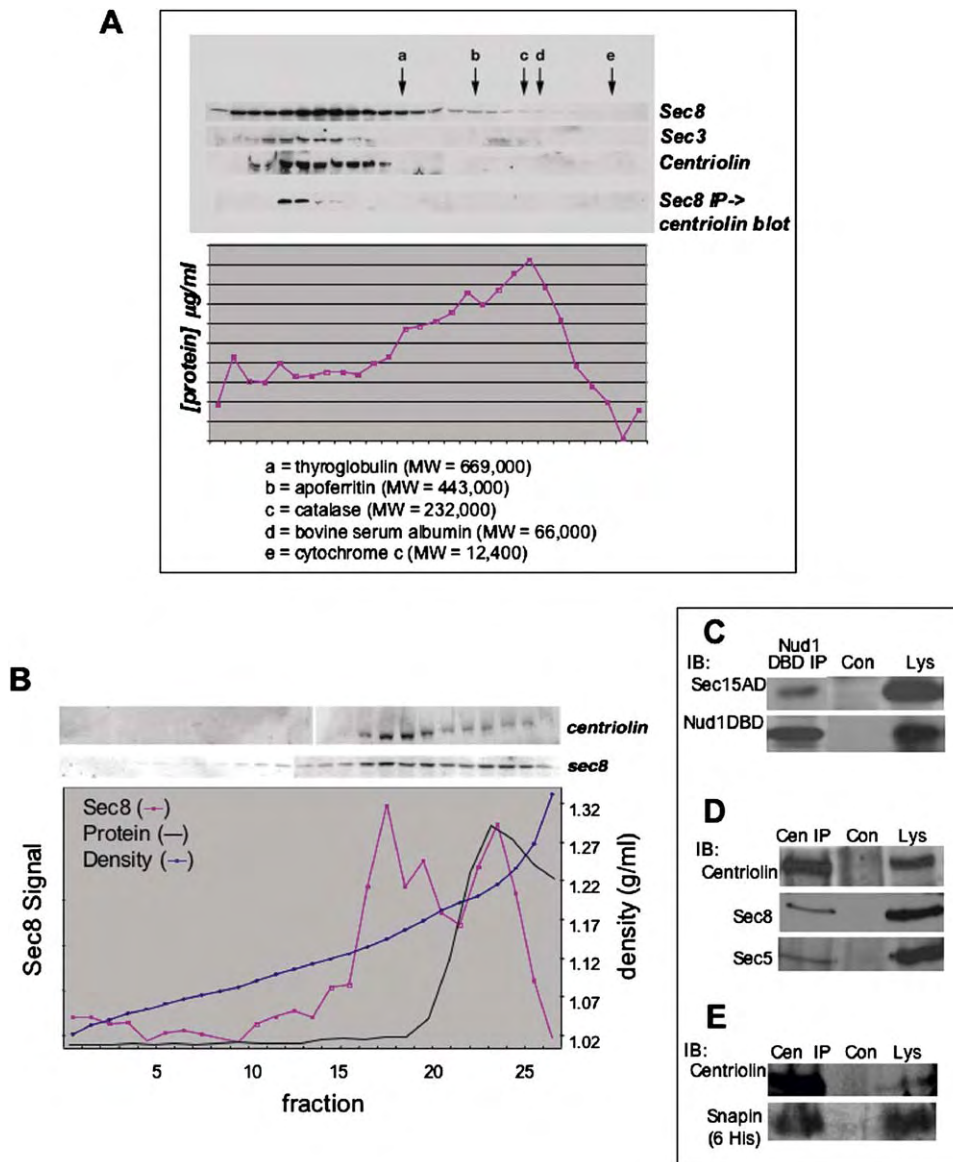
Figure 2. Centriolin Interacts with Exocyst Components and Snapin

(A) Gel filtration (Superose 6) using MDCK cell lysates shows that centriolin coelutes with peak exocyst fractions (top). Immunoprecipitation (IP) of sec8 coprecipitates centriolin. Graph, total protein profile; markers a–e are indicated.
(B) Following isopycnic centrifugation (iodixanol), centriolin comigrates in peak fractions containing sec8 (upper panels). Graph shows sec8 levels, iodixanol density, and total protein.
(C) Immunoprecipitation (IP) of Nud1-DBD (DBD antibody) pulls down sec15-AD (left). DBD, DNA binding domain; AD, activation domain; Con, control beads; Lys, lysate.
(D) Endogenous exocyst components coimmunoprecipitate with endogenous centriolin (Cen IP).
(E) Endogenous centriolin immunoprecipitates (Cen IP) overexpressed His_6-tagged snapin.

The centralspindlin components MKLP-1/CHO1/ZEN-4 (Figure 1G) and MgcRacGAP/CYK-4 (data not shown) also localized to the midbody ring and appeared earlier than centriolin during actomyosin-ring constriction. Depletion of MKLP-1 by RNAi to 18% of control levels (n = 2 experiments) prevented recruitment of centriolin to the ring (Figures 1H and 1I). In contrast, depletion of centriolin had no effect on the localization of MKLP-1 or MgcRacGAP (data not shown). These data suggested that centralspindlin anchored centriolin to the midbody ring.

Centriolin Interacts with the Exocyst Complex and the SNARE-Associated Protein Snapin and Is in Membrane-Associated Cytoplasmic Fractions

To determine the molecular function of centriolin in cytokinesis, we performed a yeast two-hybrid screen using a 120 amino acid domain of centriolin that is required for the cytokinesis function of centriolin and shares homology with budding- and fission-yeast genes (Nud1/Cdc11) involved in cytokinesis and mitotic exit (Gromley et al., 2003). A screen of approximately 12 million clones from a human testis cDNA library
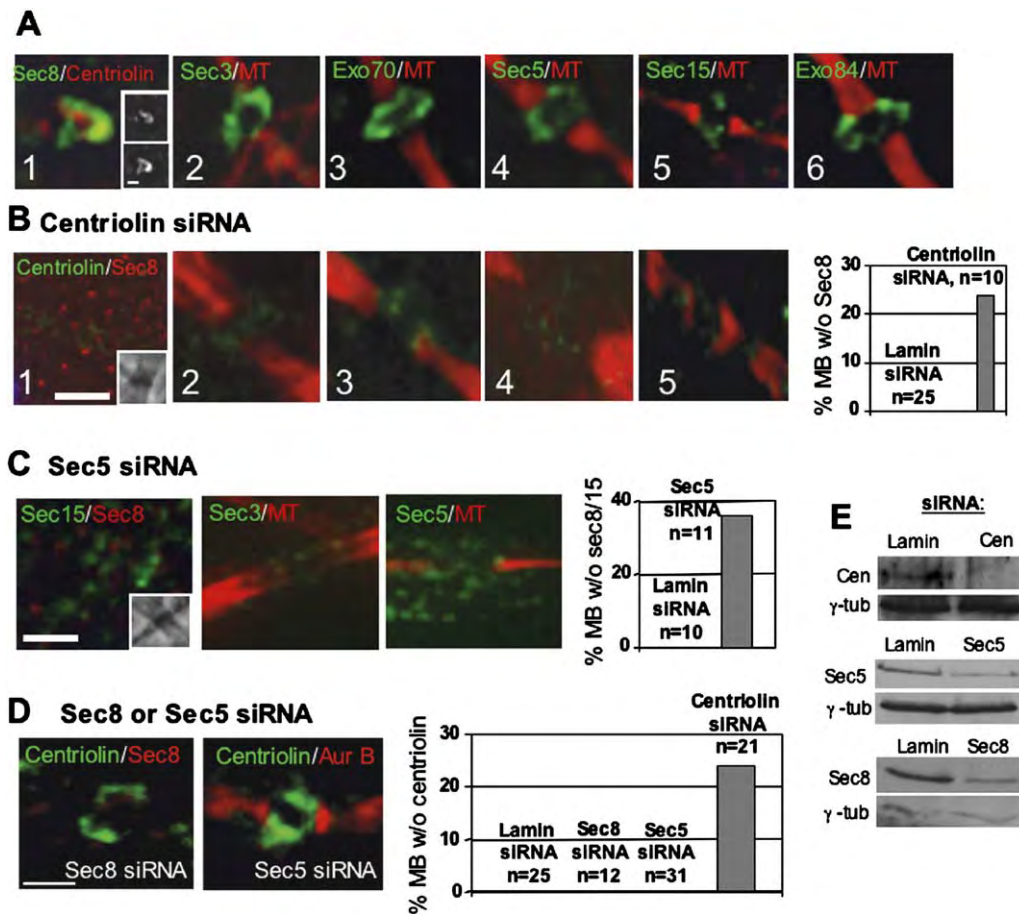
Figure 3. Exocyst Localization to the Midbody Ring Is Centriolin Dependent

(A) Immunofluorescence images of exocyst components (green) costained with centriolin antibodies (panel 1) or with anti-α-tubulin antibody (red) to visualize microtubules (MTs, panels 2–6). Panel 1 inset: top, sec8; bottom, centriolin.

(B) Cells depleted of centriolin lack midbody-associated exocyst. Images labeled as in A1–A5; B1 inset, Flemming body. Graph, percentage of midbodies (MB) without (w/o) sec8 signal following treatment with siRNAs targeting lamin A/C or centriolin; other cells have reduced levels (see text).

(C) siRNA depletion of sec5 disrupts the exocyst from midbodies costained with two exocyst proteins (C1 inset, phase) or one exocyst protein and microtubules (C2–C3). Graph, percentage of midbodies (MB) lacking sec5 staining in cells treated with lamin A/C or sec5 siRNAs.

(D) Exocyst disruption by siRNAs does not affect centriolin midbody localization. Graph, percentage of midbodies (MB) lacking centriolin stain following treatment of indicated siRNAs. Scale bar equals 1 μm (all panels).

(E) Immunoblots showing reduction of proteins targeted by siRNAs. γ-tubulin (γ-tub), loading control. Cen, centriolin.

yielded two potential interacting proteins: sec15, a member of the exocyst complex, and snapin, a SNARE-associated protein.

Additional biochemical analysis confirmed the yeast two-hybrid interactions and demonstrated that centriolin was in a large complex associated with membranes (Figure 2). The centriolin Nud1 domain fused to the DNA binding domain (DBD) and sec15 fused to the activation domain (AD) were coexpressed in the same yeast cells. Immunoprecipitation of the Nud1 fusion protein effectively coprecipitated the sec15 fusion protein (Figure 2C). To test whether other members of the exocyst complex were bound to centriolin, we immunoprecipitated endogenous centriolin from HeLa cell lysates with affinity-purified centriolin antibodies and showed that sec8 and sec5 coprecipitated (Figure 2D). Gel filtration experiments (Superose 6) using MDCK cell lysates

demonstrated that centriolin coeluted with fractions containing the exocyst complex (detected with antibodies to sec8 and sec3, Figure 2A). Centriolin was eluted as a single peak that overlapped with peaks of sec3 and sec8. We next asked if centriolin coimmunoprecipitated with the exocyst. Antibodies to sec8 were added to each of the fractions from the gel filtration column, and immune complexes were collected and probed with affinity-purified centriolin antibodies as described (Gromley et al., 2003). Centriolin was found only in fractions containing exocyst components (Figure 2A). The centriolin-containing fractions eluted earlier than the peak of sec 3 or sec8, suggesting that the exocyst fraction to which centriolin was bound was different from the cytosolic and lateral plasma-membrane fractions of the exocyst (Yeaman et al., 2004). The exocyst-centriolin fractions did not cofractionate with the
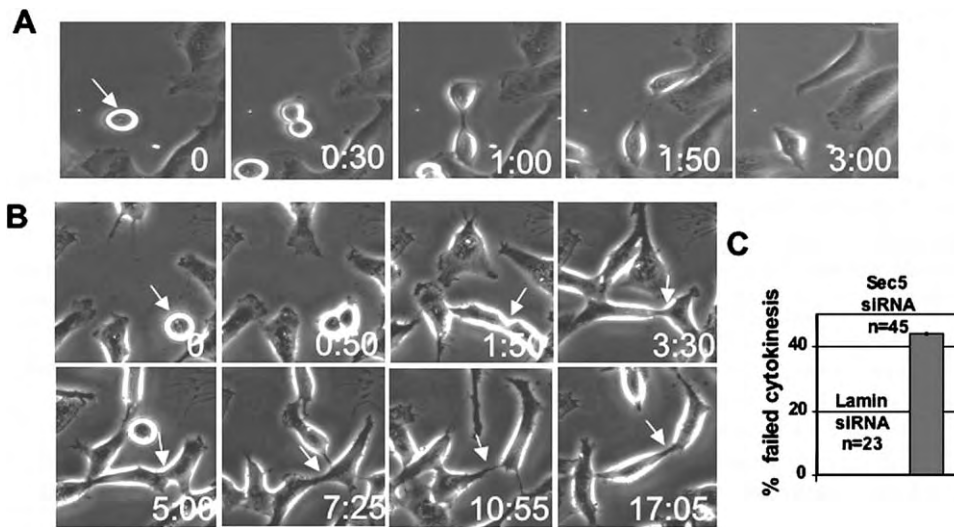
Figure 4. Exocyst Disruption Induces Cytokinesis Defects

(A) Time-lapse images of a HeLa cell treated with lamin A/C siRNAs showing a mitotic cell entering mitosis (arrow), forming a cleavage furrow, and cleaving into two separate cells in 3 hr. Time, hr:min.

(B) A cell depleted of sec5 enters mitosis (arrow), forms a cleavage furrow with normal timing (~50 min), and remains interconnected by a thin intercellular bridge for over 17 hr (panels 1:50 through 17:05).

(C) Graph shows percentage of mitotic cells that fail cytokinesis; many others are delayed (see text).

bulk of the cellular protein and eluted considerably earlier than thyroglobulin (MW 669,000) suggesting it was part of a large complex.

Since the exocyst associates with membrane vesicles, we next tested whether centriolin was also present in membranous fractions. Cell homogenates were prepared in the absence of detergent and underlain at the bottom of linear iodixanol gradients. Isopycnic centrifugation was performed, and fractions were probed for both centriolin and the exocyst component sec8. Centriolin "floated up" to fractions lighter than the cytosol having a buoyant density of $\delta \sim 1.14$ g/ml (Figure 2B). The centriolin peak cofractionated with a major peak of Sec8 that was slightly less dense than the junction-associated peak of Sec8 described previously in confluent MDCK cells ($\delta \sim 1.16$ g/ml; Yeaman et al., 2004). Little to no centriolin was observed at other positions in the gradient or in the major protein peak, suggesting that most if not all centriolin was associated with membranes. Taken together, the density gradient, immunoprecipitation, and chromatography data support the conclusion that centriolin associates with the exocyst in a very large complex bound to cellular membranes. The yeast two-hybrid interaction between centriolin and the low-abundance protein snapin was confirmed by showing that endogenous centriolin coimmunoprecipitated a His_6-tagged snapin fusion protein expressed in HeLa cells (Figure 2E) and by the centriolin-dependent midbody localization of snapin (see below).

The Exocyst Complex Colocalizes with Centriolin at the Midbody Ring
Further support for the centriolin-exocyst interaction was obtained by showing that exocyst-complex components localized to the midbody ring with centriolin.

HeLa cells were colabeled with antibodies against one of several exocyst components (sec3, sec5, sec8, sec15, exo70, or exo84) and either microtubules or centriolin (Figure 3A). We found that all these exocyst components localized to the midbody ring during cytokinesis and formed a ring-like structure similar to that seen for centriolin. In fact, double-stained images revealed considerable overlap between sec8 and centriolin, indicating that they were part of the same structure (Figure 3A, panel 1). We also showed that a myc-tagged form of sec8 localized to the midbody ring when expressed in HeLa cells (Figure S1), confirming the localization seen with antibodies directed to the endogenous protein.

Midbody Localization of the Exocyst Is Disrupted in Cells Depleted of Centriolin
We next tested whether centriolin was required for midbody-ring localization of the exocyst. siRNA-mediated depletion of centriolin resulted a in ~70% reduction in centriolin protein levels and complete loss of midbody staining in 24% of cells compared with control cells treated with lamin siRNA (Figures 3B and 3E). Immunofluorescence quantification of midbody signals performed as in our previous studies (Gromley et al., 2003) demonstrated that many of the remaining centriolin-depleted cells had lower levels of midbody staining than controls (48%, n = 23 cells), bringing the total percentage of midbody depleted cells to 72%. Cells that lacked detectable midbody-associated centriolin usually lacked midbody labeling of sec8 (10/10, Figure 3B, panels 1 and 6). Although other exocyst components could not be costained with centriolin because all were detected with rabbit antibodies like centriolin, all were lost from or reduced at midbodies in centriolin-depleted cells (Figure 3B, panels 2–5). For example,
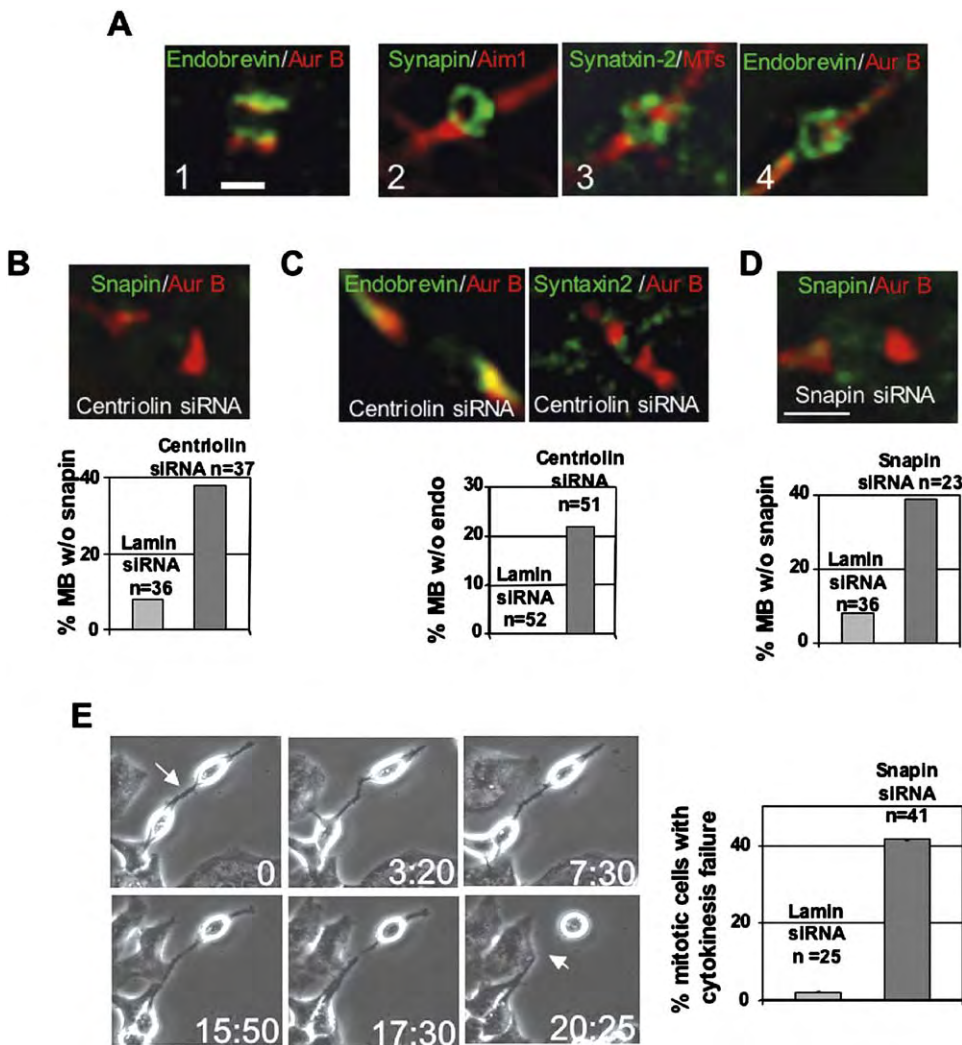
Figure 5. Centriolin siRNA Mislocalizes Midbody-Ring-Associated SNAREs and Snapin, which Disrupts Cytokinesis When Depleted

(A) Endobrevin/VAMP8 (1) localizes adjacent to the midbody ring when snapin is on the ring (2). Later, when the midbody diameter is thin (0.5–1 μm), endobrevin/VAMP8 and syntaxin-2 localize to the ring (3 and 4).

(B) Centriolin-depleted cell shows loss of snapin from the midbody ring (green). Graph, percentage of midbodies lacking snapin after siRNA depletion of proteins.

(C) Centriolin-depleted cells lose SNARE proteins from the midbody ring. Graph, percentage of midbodies lacking endobrevin/VAMP8 staining after indicated siRNAs treatments. Endo, endobrevin.

(D) Snapin-depleted cells show loss of snapin from the midbody ring. Graph, percentage of midbodies lacking snapin after indicated siRNA treatments.

(E) A snapin-depleted cell in cytokinesis (0) remains connected by a thin intercellular bridge for >17 hr before separating (20:25) (time, hr:min). Graph, percentage of mitotic cells that failed cytokinesis.

Exo84 was undetectable at midbodies in 22% of centriolin-depleted cells (n = 9 cells) or had levels below the lowest control midbody staining in 42% of centriolin-depleted cells (n = 19 cells). Significant reduction in midbody staining of centriolin and other exocyst components was observed with a second siRNA targeting a different centriolin sequence (Gromley et al., 2003) (data not shown).

To test whether centriolin was dependent on the exocyst complex for localization to the midbody, we initially targeted sec5 for siRNA depletion. Recent studies showed that mutants of sec5 in *D. melanogaster* dis-

rupted exocyst function (Murthy and Schwarz, 2004) and that RNAi-mediated depletion of sec5 inhibited exocyst-dependent processes in vertebrate cells (Prigent et al., 2003). We found that depletion of sec5 resulted in loss of midbody-associated sec5 as well as other exocyst components, including sec3, sec8, and sec15 (Figures 3C and 3E). These results show that sec5 depletion disrupts midbody-ring localization of the exocyst. In contrast, neither sec5 nor sec8 loss from the midbody affected the association of centriolin with the midbody ring (Figures 3D and 3E). These data demonstrate that centriolin is required for midbody localiza-
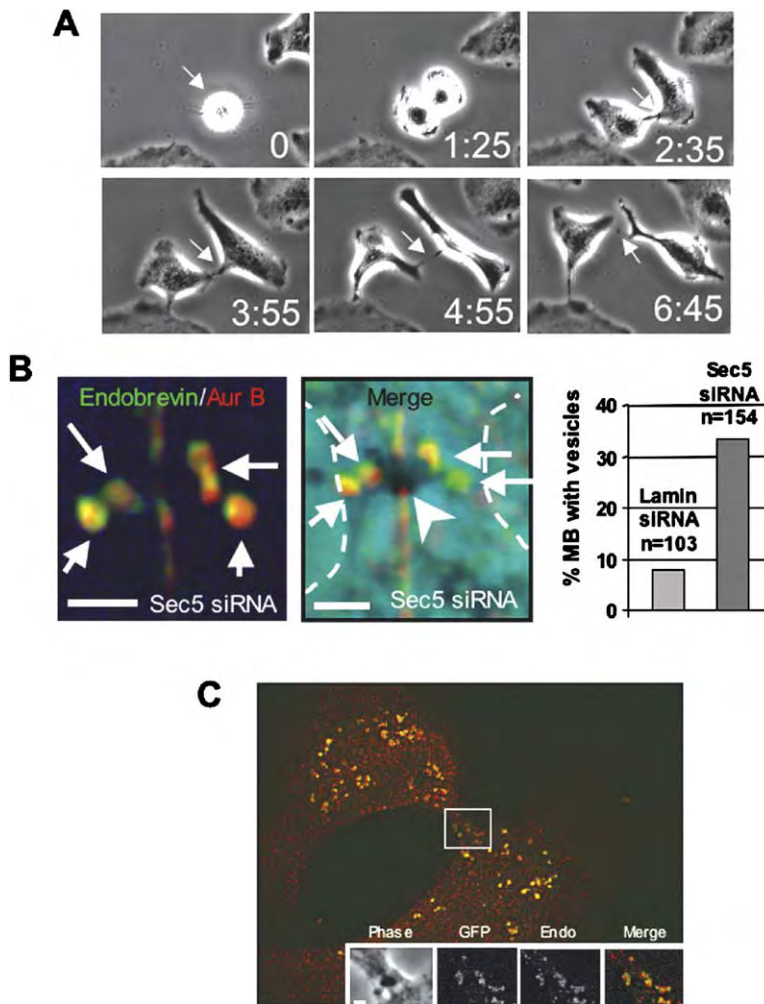
Figure 6. Disruption of the Exocyst Results in Accumulation of Secretory Vesicles at the Midbody Ring

(A) A mitotic cell (0, arrow) treated with BFA exits mitosis and forms a cleavage furrow with normal timing but arrests with a thin intercellular bridge that connects the two daughters (panels 2:35 through 6:45).

(B) sec5 siRNA-treated cells accumulate endobrevin/VAMP8-containing vesicle-like structures (arrows) at the Flemming body (arrowhead, panel 2). Dotted lines, plasma membrane. Graph, percentage of cells with endobrevin/VAMP8 vesicles at the midbody following indicated siRNA treatments. Scale bars, 2 μm.

(C) Endobrevin/VAMP8 (green) localizes to luminal-GFP secretory vesicles (red). Box at midbody is enlarged in insets. Endo, endobrevin/VAMP8.

tion of the exocyst, while localization of centriolin appears to be independent of the exocyst.

Disruption of the Exocyst Causes Failure at the Final Stages of Cytokinesis

Localization of the exocyst to the midbody and its interaction with centriolin suggested that the complex might play a role in cytokinesis. To examine this, we disrupted the midbody-associated exocyst using siRNAs targeting sec5 and examined cytokinesis by time-lapse imaging over a 20 hr time period. We found that over half the cells exhibited severe cytokinesis defects, including failure in the final abscission step (42%, Figures 4B and 4C, Movie S3) and delays during cytokinesis (24%, n = 18) compared with control lamin siRNA-treated cells (Figures 4A and 4C, Movie S2). Some cells remained interconnected by thin cytoplasmic bridges (Figure 4B, panel 17:05 and Movie S3) and sometimes entered one or more additional rounds of mitosis while still connected to their partner cells. Sec5-depleted cells viewed for an additional 24 hr showed a similar level of cytokinesis defects (data not shown), suggesting that nearly all cells in the culture experienced cytokinesis problems over time. Cytokinesis defects were

also observed when the exocyst was disrupted by siRNA depletion of sec15 and sec8 (data not shown). Cells remained healthy, as no differences in cell morphology or mitochondrial function were observed. These data show that disruption of the exocyst produces late-stage cytokinesis defects similar to centriolin (Gromley et al., 2003) and demonstrates a requirement for the exocyst in the final stages of animal cell cytokinesis.

Snapin and SNARE Components Localize to the Midbody Ring in a Centriolin-Dependent Manner

Snapin was originally considered to be a neuron-specific protein, but recent studies demonstrated that it is also expressed in nonneuronal cells (Buxton et al., 2003). Snapin may facilitate assembly of SNARE complexes and may define a limiting step in vesicle fusion mediated by PKA phosphorylation (Chheda et al., 2001). Although the role of snapin in neurotransmission has been questioned (Vites et al., 2004), recent results indicate that it is essential for this process (Thakur et al., 2004). The role of snapin in cytokinesis is currently unknown. Using previously characterized antibodies to snapin (Thakur et al., 2004), we demonstrated that the
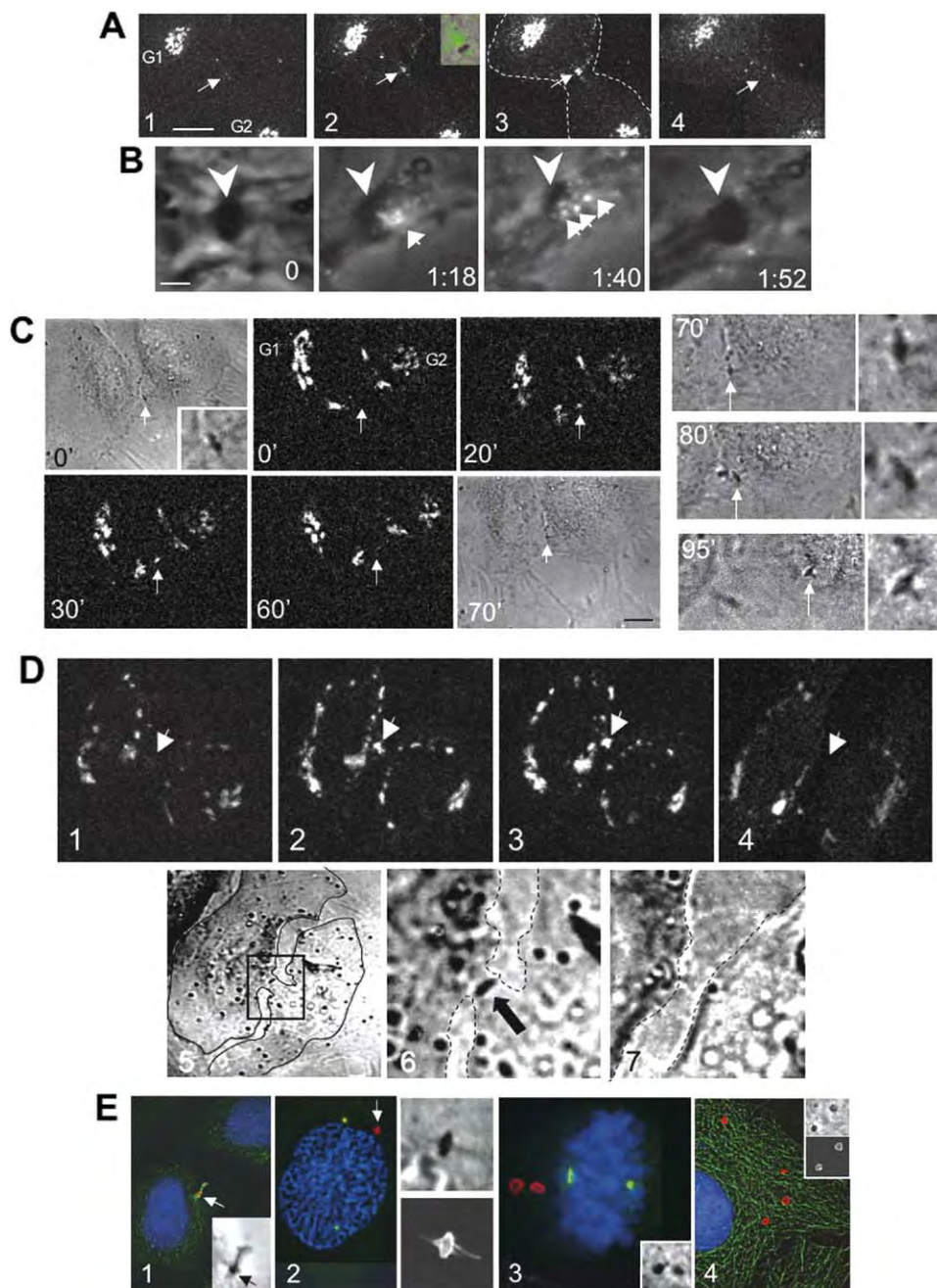
Figure 7. Asymmetric Delivery of Secretory Vesicles to One Side of the Flemming Body Is Followed by Abscission at This Site

(A) A dividing HeLa cell expressing luminal GFP accumulates secretory vesicles on one side of the Flemming body (arrows in 2 and 3, inset). In panel 1, most luminal-GFP signal is in Golgi complexes (G1 and G2). The signal appears transiently at one side of the midbody (2 and 3, arrows; Movie S5) and is lost, although Golgi signal remains (4). Scale bar in panel 1, 10 μm.

(B) Higher-magnification images of another cell (see Movie S6) showing unidirectional delivery of luminal-GFP-containing vesicles from one nascent daughter cell to one side of the Flemming body (arrowhead). GFP vesicles move to the Flemming body from the cell on the right (1:18 and 1:40, arrows; see Movie S6) and quickly disappear (1:52), presumably due to vesicle fusion with the plasma membrane and diffusion of the signal into the extracellular space. Phase and GFP signals are overlaid. Time, hr:min. Scale bar in panel 1, 1 μm.

(C) Lum-GFP vesicle delivery to the Flemming body (0'–30', arrows) followed by signal loss (60', at arrow) and abscission (80' and 95'). Phase-contrast images were taken after disappearance of GFP signal. Enlargements of Flemming body are shown to the right of each low-magnification image in 70'–95'. Scale bar at 70': 10 μm for 0'–95' and 2 μm for enlargements in 70'–95'.

(D) Lum-GFP vesicle delivery to one side of the midbody (panels 1–3) followed by disappearance of the GFP signal (panel 4) and abscission (loss of intercellular bridge, panels 5–7, arrows). The box in panel 5 is enlarged in panel 6. Solid and dotted lines show cell boundaries.

(E) Postmitotic cell (1) showing microtubules (green, GT335 antibody) of the intercellular bridge (phase-contrast image, inset) attached to one of the two daughter cells; no detectable midbody microtubules are seen on the other cell. Microtubules are on both sides of the midbody ring (arrow, red, MKLP-1) and Flemming body (inset, phase), showing that the midbody with attached microtubules was delivered to one daughter cell. Prophase HeLa cell (2) with condensing chromatin (blue) and two centrosomes (green) has a midbody ring and lateral material

protein localized to the midbody ring at the same time as the exocyst and shortly after centriolin (Figure 5A, panel 2).

Previous immunofluorescence studies showed that the v-SNARE endobrevin/VAMP8 and t-SNARE syntaxin-2 were enriched in the region of the midbody flanking the Flemming body and coincident with microtubules and Aurora B staining (Low et al., 2003). Using the same antibodies, we confirmed the localization pattern of endobrevin/VAMP8 (Figure 5A, panel 1) and syntaxin-2 (data not shown). Very late in cytokinesis, the intercellular bridge narrows to ~0.5 μm, and microtubule bundles are reduced in diameter to 0.2–0.5 μm. At this time, endobrevin/VAMP8 and syntaxin-2 joined centriolin, snapin, and the exocyst at the midbody ring (Figure 5A, panels 3 and 4). siRNA depletion of centriolin eliminated the midbody-ring localization of snapin (>35% of cells, Figure 5B), endobrevin/VAMP8 (>20% of cells, Figure 5C), and syntaxin-2 (Figure 5C). Of the remaining cells, 24% and 36% showed midbody staining levels below those of controls for snapin (n = 22) and endobrevin/VAMP8 (n = 25), respectively. As shown earlier, midbody-ring integrity was not compromised under these conditions, as MKLP-1 and MgcRacGAP remained at this site in cells with reduced centriolin. These results indicated that centriolin was required for midbody-ring localization of v- and t-SNARE proteins and the SNARE-associated protein snapin.

Snapin Depletion Mislocalizes the Protein from the Midbody and Induces Cytokinesis Defects

Midbodies in 41% of snapin-depleted cells showed no detectable snapin staining (Figure 5D). Time-lapse imaging over a 22 hr period showed that 40% of snapin-depleted cells experienced late-stage cytokinesis failure (Figure 5E, Movie S4). Other cells showed long delays and often remained connected by a thin intercellular bridge (data not shown). When cultures were imaged for an additional 24 hr, we observed multicellular syncytia resulting from multiple incomplete divisions and additional individual cells undergoing cytokinesis failure. This suggested that most cells in the population ultimately failed cytokinesis and that some failed multiple times. Occasionally, cells separated when one of the attached daughters re-entered mitosis, possibly due to tensile forces generated by cell rounding during mitosis (Figure 5E, Movie S4). These results demonstrated that snapin was necessary for abscission and suggested that it functioned by anchoring SNARE complexes at the midbody.

Disruption of the Exocyst Results in Accumulation of Secretory Vesicles at the Midbody

We next tested whether the late-stage cytokinesis defects observed in this study resulted from changes in membrane trafficking to the midbody. As a first test of this idea, we used brefeldin A, which disrupts cytokine-

sis in *C. elegans* presumably due to inhibition of post-Golgi secretory-vesicle trafficking (Skop et al., 2001). In HeLa cells treated with brefeldin A, we observed late-stage cytokinesis defects (Figure 6A) that were similar to those observed following depletion of centriolin. Many cells were delayed in or failed cytokinesis (n = 9/13 cells in two separate experiments). This suggested that post-Golgi vesicle trafficking was involved in late-stage cytokinesis events in vertebrate cells, although brefeldin A is known to affect other membrane-trafficking pathways (Antonin et al., 2000).

Based on the localization of the exocyst to the midbody ring, we reasoned that the vesicle-tethering function of the complex might be operating at this site to facilitate fusion of v-SNARE-containing vesicles at the late stages of cytokinesis. To test this idea, we depleted cells of sec5 to disrupt exocyst complexes and examined the localization of v-SNARE (endobrevin/VAMP8) containing vesicles. We observed a collection of small, spherical endobrevin/VAMP8-containing structures resembling vesicles at the midbody (Figure 6B, panel 1, arrows) that were positioned around the phase-dense Flemming body (Figure 6B, arrowhead, panel 2). Although these structures were occasionally seen in control lamin A/C siRNA-treated cells, they were significantly increased in sec5-depleted cells (Figure 6B, graph).

To determine whether the endobrevin/VAMP8-containing structures were secretory vesicles, we used a more specific marker for the secretory pathway. We expressed a GFP-tagged construct containing an amino-terminal signal peptide that targets the protein to the lumen of the ER (lum-GFP) (Blum et al., 2000) and lacks retention and retrieval motifs, so it would not be expected to target to endosomes, multivesicular bodies, or lysosomes. The lum-GFP was efficiently secreted from nondividing MDCK cells following a 19°C *trans*-Golgi network block and release from the block in the presence of protein-synthesis inhibitors (C.Y., unpublished data). When we expressed lum-GFP, numerous GFP-containing vesicles were observed in the cytoplasm. Following fixation and staining for endobrevin/VAMP8, we found that most of the endobrevin/VAMP8 vesicles colabeled with lum-GFP throughout the cytoplasm (Figure 6C) and within the intercellular bridge during late stages of cytokinesis (Figure 6C, insets). This observation demonstrates that the v-SNARE-containing vesicles that accumulated following disruption of the exocyst are secretory vesicles, an observation similar to that seen in studies in exocyst mutants of *S. cerevisiae* where vesicles dock normally but fail to fuse with the plasma membrane (Guo et al., 2000)

Asymmetric Delivery of Secretory Vesicles to the Midbody Is Followed by Abscission

At early stages of cytokinesis, we observed numerous GFP-labeled secretory vesicles in Golgi complexes and

stained with MKLP-1 (arrow, red) and in enlargement (bottom right); the Flemming body with flanking material is enlarged at upper right. Metaphase cell (3) with two midbody rings stained for MKLP-1 (red). Inset, two Flemming bodies corresponding to the two MKLP-1-stained structures. Centrosomes, green; DNA, blue. Interphase cell (4) showing four MKLP-1-stained midbody rings (red). Two are enlarged in lower inset and colocalize with phase-dense Flemming bodies (upper inset). DNA, blue; microtubules, green.

cell bodies of nascent daughter cells but few within intercellular bridges (Figure 7A, panel 1). However, at a late stage of cytokinesis when the intercellular bridge narrowed to a diameter of ~ 2 μm and the midbody microtubule bundle was reduced to a diameter of 0.5–1 μm, GFP secretory vesicles accumulated in the intercellular bridge near the midbody ring (Figure 7A, Movie S5). Higher-magnification imaging of another cell at a similar cell-cycle stage revealed labeled secretory vesicles moving suddenly and rapidly (within 20 min) from the cell bodies into the intercellular bridge and up to the midbody ring (Figure 7B, Movie S6). In 11/11 cells, the vesicles were delivered primarily if not exclusively from one of the nascent daughter cells (Figure 7B, center panels). Vesicles packed into the region adjacent to the phase-dense Flemming body (Figure 7B, panels 2 and 3, large arrowhead; Movie S6). Within 20 min, the GFP signal disappeared (Figure 7B, last panel and Figure 7A, last panel), suggesting that the vesicles fused with the plasma membrane, releasing the GFP signal into the extracellular space where it was free to diffuse. Loss of the GFP signal was not due to photobleaching because GFP-labeled vesicles in cell bodies adjacent to the intercellular bridge and in the Golgi complex retained the signal. We next examined the relationship between vesicle delivery to the midbody and abscission. We found that, shortly after the GFP signal was lost from the midbody region, the cell cleaved on the side of the Flemming body that received the GFP vesicles (6/6 cells from four experiments, Figure 7C). The cell on the opposite side received the Flemming body (Figure 7C, 70'–95' and Figure 7D). In some cases, the Flemming body moved around rapidly after abscission on the cell surface (Movie S7), suggesting that the structure was not anchored at a discrete point on the new daughter cell. Postdivision midbodies contained multiple midbody-ring components and retained microtubules from both sides of the midbody ring (Figure 7E, panel 1). They persisted for some time after abscission, consistent with previous results (Mishima et al., 2002), and were often present in multiple copies, suggesting that they were retained through several cell cycles (Figure 7E, panels 2–4). These structures were seen on $\sim 35\%$ of HeLa cells and often retained features of the Flemming body and midbody ring, including MKLP-1 staining, Aurora B staining, phase-dense Flemming bodies, and localization to the plasma membrane (Figure 7E, data not shown). This suggested that supernumerary midbodies represent structures from previous divisions similar to the bud scars observed in yeast (Chen and Contreras, 2004).

Discussion

A Model for the Final Stage of Cytokinesis
This study defines several distinct molecular and structural steps during the late stages of cytokinesis (Figure 8). During cleavage-furrow ingression, MKLP-1 and MgcRacGAP arrive at the midbody ring (Figure 8A). When the intercellular bridge forms, centriolin localizes to the ring (Figure 8B), followed by snapin and exocyst proteins (Figure 8C). When the diameter of the midbody microtubule bundle and the intercellular bridge are reduced to ~ 0.5–1 μm, endobrevin/VAMP8 (v-SNARE) and syntaxin-2 (t-SNARE) move to the midbody ring. The v-SNAREs are part of secretory vesicles that move asymmetrically into the intercellular bridge predominantly from one nascent daughter cell; binding to v-SNAREs may incorporate t-SNAREs into this organization. The vesicles pack into the area adjacent to the ring and appear to fuse, releasing their contents into the extracellular space (lum-GFP, Figures 8D and 8E). Vesicle fusion with the plasma membrane may be initiated near the midbody ring where v- and t-SNAREs are localized. This could be followed by additional fusion events between vesicles and the plasma membrane as well as vesicle-vesicle fusion events (homotypic) mediated by SNAP23/25, a v-SNARE involved in compound exocytosis (Takahashi et al., 2004) (Figures 8F and 8G). Abscission then occurs at the site of vesicle fusion, and the entire midbody remains with the daughter cell opposite the fusion site (Figure 8H). Abscission could be triggered by arrival of v- and t-SNAREs at the midbody ring; release of SNAP23/25 from lipid rafts (Takahashi et al., 2004; Takeda et al., 2004); phosphorylation of snapin by PKA, which mediates its binding to the t-SNARE complex (Buxton et al., 2003; Chheda et al., 2001); or another event. Dynamic movement of the postabscission midbody ring suggests connections to motile forces within the cell, although this remains to be determined.

Asymmetric Delivery of Secretory Vesicles Marks the Site of Abscission
It is remarkable that secretory vesicles loaded with luminal GFP move into the intercellular bridge from only one of the two prospective daughter cells. The mechanism of this asymmetric vesicle delivery is unknown. It is tempting to speculate that a signal, negative or positive, emanates asymmetrically from one centrosome in the dividing cell. Centrosomes in the two prospective daughter cells are different in that one was "born" from the older centriole in the previous cell division during the centrosome duplication process (Doxsey, 2001). Consistent with this idea is the asymmetric spindle-pole body (SPB) localization of budding- and fission-yeast proteins that control mitotic exit and cytokinesis (Doxsey et al., 2005; Grallert et al., 2004; Molk et al., 2004). In S. pombe, inhibitors of mitotic exit (Cdc16p and Byr4p) localize to the "old" SPB while activators of mitotic exit (Cdc7p and presumably Sid1p and Cdc14p) localize to the new SPB (Grallert et al., 2004). The relevance of this localization in both yeasts is still unknown. Further studies will be required to determine the role of centrosome protein asymmetry in the unidirectional delivery of secretory vesicles and abscission in animal cells. It has been suggested that the mother centriole moves to the intercellular bridge in telophase cells to coordinate the final steps in cytokinesis (Piel et al., 2001), although this was not consistently observed in this study (data not shown) or another that investigated several cell lines (RPE-1, Ptk-1, CV-1, NRK-52E; A. Khodjakov, personal communication).

The final stages of cytokinesis in animal cells share features with cell division in higher plants. Higher plant cells cannot divide using an actomyosin-based cleavage furrow due to the presence of a nonpliant cell wall,
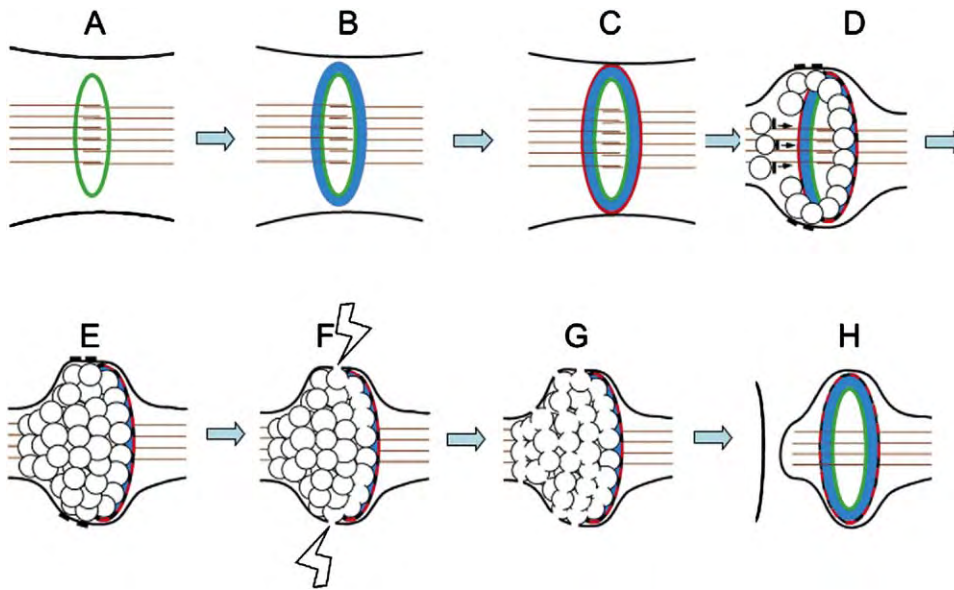
Figure 8. Model Depicting Vesicle-Mediated Abscission during Cytokinesis

(See text for details.)

(A) MKLP-1 and MgcRacGAP (green) arrive at midbody ring after cleavage furrowing has progressed. Microtubules, brown; plasma membrane, upper and lower lines.

(B and C) Centriolin moves to ring ([B], blue) and anchors sec15, other exocyst components, and snapin ([C], red).

(D) When midbody microtubules are reduced and the membrane constricted, v- and t-SNAREs ([D], black) move to the midbody ring from one prospective daughter cell. v-SNAREs presumably move with vesicles and bind there in a centriolin-dependent manner; t-SNAREs on the plasma membrane could bind through v-SNAREs.

(E) Vesicles heterogeneous in diameter pack asymmetrically into the intercellular bridge adjacent to the midbody ring.

(F and G) Vesicles adjacent to the ring containing SNARES and exocyst fuse with the plasma membrane (F) as well as at other plasma-membrane sites and with one another (G).

(H) Abscission follows at the site of membrane fusion, and the midbody is retained by the daughter cell opposite the fusion site. The released midbody ring contains multiple midbody-ring proteins and usually retains microtubule bundles from both sides of the ring. (In this model, the apparent "layering" of components is a simplification to depict arrival of different components at the midbody.)

so they accomplish cell division by constructing a new membrane at the division plane, called the cell plate, that is independent of the plasma membrane and is established by microtubule-dependent delivery and fusion of vesicles at this site (Albertson et al., 2005; Finger and White, 2002; Jurgens, 2005). Our data show that the coordinated delivery of vesicles to the midbody ring during the late stages of cytokinesis is also required for the final stages of cell division in animal cells. However, we still do not understand the mechanism of secretory-vesicle delivery to the midbody, the role of microtubules in this process, or the precise contribution of vesicle transport and fusion to abscission. The presence of vesicles with heterogeneous diameters adjacent to the midbody ring prior to abscission is consistent with a model in which some vesicles fuse together prior to fusion with the plasma membrane. This would be analogous to the cell plate in plant cells. The endocytic pathway also appears to play a role in cell cleavage as components (dynamin, FIP3, Rab11) and compartments (endosomes) involved in this pathway affect the late stages of cytokinesis (Thompson et al., 2002; Wilson et al., 2005). Recycling endosomes have been shown to move from both prospective daughter cells to the midbody during cytokinesis then return to the daughter-cell cytoplasm (Wilson et al., 2005). It is still

unclear how recycling endosomes participate in abscission and how the bidirectional movement of endosomes into the intercellular bridge is related to the unidirectional movement of secretory vesicles to this site in our study.

Structure and Persistence of the Midbody Ring
We have shown that many proteins localize to the midbody ring and that the phase-dense Flemming body is also organized into the shape of a ring. This is consistent with earlier ultrastructural studies that describe cytoplasmic channels coursing through the central midbody (Mullins and Biesele, 1977). The ring structure bears a resemblance to bud scars of *S. cerevisiae,* which serve as markers for longevity (Chen and Contreras, 2004). The midbody ring in animal cells is inherited by the daughter cell that lies opposite the site of vesicle delivery and appears to persist, as it is often seen in mitotic cells prior to cytokinesis and found in multiple copies in interphase cells (Figure 7E) (Mishima et al., 2002). Shortly after abscission, the midbody ring contains microtubules that extend from both sides of the ring. This suggests that dissolution of microtubule bundles adjacent to the midbody ring is not an absolute requirement for the final stage of cytokinesis but rather that abscission can result in transfer of the entire mid-

body and associated microtubules into one daughter cell.

Experimental Procedures

Cell Culture and Transfections
The cells used primarily in this study were diploid, telomerase-immortalized human RPE cells (hTERT-RPE-1s, Clontech Laboratories, Inc.) (Morales et al., 1999) and HeLa cells. All cells were grown as previously described (American Type Culture Collection). HeLa cells were transfected as previously described (Lipofectamine, Invitrogen).

Immunofluorescence
Cells were prepared for immunofluorescence, imaged, and deconvolved (Metamorph, Universal Imaging Corp.) using either formaldehyde, formaldehyde followed by methanol, or methanol alone as previously described (Dictenberg et al., 1998). All immunofluorescence images are two-dimensional projections of three-dimensional reconstructions to ensure that all stained material was visible in two-dimensional images. Quantification of signals produced by immunofluorescence staining for various midbody antigens was performed as described for centrosome protein quantification in our earlier studies (Gromley et al., 2003).

Antibodies
Antibodies to the following proteins were used: sec3, sec5, sec8, sec10, exo70, exo84, and sec15 (Yeaman, 2003); centriolin (Gromley et al., 2003); α-tubulin, γ-tubulin, α-His$_6$, and α-myc (Sigma-Aldrich); Aurora B (Transduction Laboratories); MKLP-1, GAL4 transactivation domain (AD), and GAL4 DNA binding domain (DBD) (Santa Cruz Biotechnology, Inc.); and GT335 for stabilized microtubules (Gromley et al., 2003).

Yeast Two-Hybrid Screen
Yeast two-hybrid library screens were performed following the manufacturer's instructions using a human testis Matchmaker Pre-Transformed Two-Hybrid Library (Matchmaker GAL4 Yeast Two-Hybrid System, Clontech). False positives were eliminated by mating each clone with strains expressing either lamin C or the DNA binding domain alone and plating on quadruple dropout media.

siRNAs
Two siRNAs targeting centriolin and one targeting lamin A/C were used as described (Gromley et al., 2003). Additional siRNAs targeted nucleotides in the following proteins: MKLP-1 (189–207), sec5 (260–278), sec8 (609–627), and snapin (312–330). Cells were examined 24–48 hr after siRNA treatment. siRNAs were used at 10–50 nM, and Lipofectamine was the delivery agent (Gromley et al., 2003).

Brefeldin A Treatment
HeLa cells were treated with 5–10 μg/ml brefeldin A (Sigma-Aldrich) and imaged.

Immunoprecipitations
Antibodies to centriolin or exocyst were added to hTERT-RPE cell extracts and incubated at 4°C overnight. The lysis buffer included 50 mM Tris-HCl (pH 7.5), 10 mM Na$_2$HPO$_4$ (pH 7.2), 1 mM EDTA, 150 mM NaCl, 1% IGEPAL CA-1630, and protease inhibitors (Mini tablets, Roche Diagnostics, Mannheim, Germany). Superose 6 samples were incubated with antibodies to sec3 and sec8, bound to protein A/G beads (Santa Cruz Biotechnology, Inc.) at 4°C for 2 hr (Yeaman, 2003), and exposed to SDS-PAGE and immunoblotting (Harlow and Lane, 1988).

Time-Lapse Imaging
Time-lapse imaging of cytokinesis was performed using a widefield microscope (Gromley et al., 2003), and images were taken every 5 min for 18–24 hr. For luminal-GFP-expressing cells (Figure 7B), two concurrent time-lapse programs were used (GFP, phase contrast), and images were taken every 2 min for 3–4 hr. A Perkin-nElmer spinning-disc confocal microscope with an UltraVIEW CSU-10 head was used for Figures 7A, 7C, and 7D. Images were taken every 5 min and captured on an ORCA-AG cooled CCD camera. Images of GFP-GAPCenA-expressing cells were taken every 10 min on a Zeiss Axiophot microscope equipped with a Hamamatsu digital camera. Mitochondria function was assessed by Mitotracker staining (Molecular Probes).

Exocyst Fractionation
For isopycnic centrifugation, membrane compartments containing exocyst fractions were prepared as described (Grindstaff et al., 1998; Yeaman, 2003). For size-exclusion chromatography, cells were extracted with MEBC buffer (0.5% Nonidet P-40, 50 mM Tris-HCl [pH 7.5], 100 mM NaCl) containing protease inhibitors (0.1 mM Na$_3$VO$_4$; 50 mM NaF; 1 mM Pefabloc [Boehringer Mannheim]; and 10 μg/ml each of leupeptin, antipain, chymostatin, and pepstatin A) for 10 min at 4°C. Lysates were first sedimented in a Microfuge (Beckman Instruments, Fullerton, California) for 10 min and then for 30 min at 100,000 × g, passed through a 0.22 μm filter (Millipore), and loaded on a Superose 6 HR 10/30 column (200 μl, 10 mm × 30 cm; Pharmacia Biotech, Inc.) equilibrated in MEBC buffer and 1 mM dithiothreitol with 0.1 mM Pefabloc. Proteins were eluted (0.3 ml/min) at 17°C in 0.5 ml fractions, the concentration of protein in the fractions was determined, and the fractions were used for various assays (fractions 7–30).

Supplemental Data
Supplemental Data include one figure and seven movies and can be found with this article online at http://www.cell.com/cgi/content/full/123/1/75/DC1/.

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JCB Report

Pericentrin forms a complex with intraflagellar transport proteins and polycystin-2 and is required for primary cilia assembly

Agata Jurczyk,[1] Adam Gromley,[1] Sambra Redick,[1] Jovenal San Agustin,[2] George Witman,[2] Gregory J. Pazour,[1] Dorien J.M. Peters,[3] and Stephen Doxsey[1]

[1]Department of Molecular Medicine and [2]Department of Cell Biology, University of Massachusetts Medical School, Worcester, MA 01605
[3]Leiden University Medical Center, Department of Human Genetics, 2333 AL Leiden, Netherlands

Primary cilia are nonmotile microtubule structures that assemble from basal bodies by a process called intraflagellar transport (IFT) and are associated with several human diseases. Here, we show that the centrosome protein pericentrin (Pcnt) colocalizes with IFT proteins to the base of primary and motile cilia. Immunogold electron microscopy demonstrates that Pcnt is on or near basal bodies at the base of cilia. Pcnt depletion by RNA interference disrupts basal body localization of IFT proteins and the cation channel polycystin-2 (PC2), and inhibits primary cilia assembly in human epithelial cells. Conversely, silencing of IFT20 mislocalizes Pcnt from basal bodies and inhibits primary cilia assembly. Pcnt is found in spermatocyte IFT fractions, and IFT proteins are found in isolated centrosome fractions. Pcnt antibodies coimmunoprecipitate IFT proteins and PC2 from several cell lines and tissues. We conclude that Pcnt, IFTs, and PC2 form a complex in vertebrate cells that is required for assembly of primary cilia and possibly motile cilia and flagella.

Introduction

Centrosomes serve as microtubule-organizing centers in interphase and mitotic cells and play a role in cytokinesis and cell cycle progression (Doxsey, 2001). They are also the precursors of primary cilia, nonmotile sensory organelles found on most vertebrate cells. Ciliary dysfunctions are associated with several human diseases (Pazour and Rosenbaum, 2002; Rosenbaum and Witman, 2002). Primary cilia in vertebrate cells appear to arise from the mother centriole of the centrosome within a membrane sheath, which forms from cytoplasmic vesicles and ultimately fuses with the plasma membrane (Sorokin, 1968). The intimate relationship between the centrosome and the primary cilium suggests that functions and components may be shared between these structures.

Primary cilia assembly occurs by a process called intraflagellar transport (IFT) (Kozminski et al., 1993; Pazour and Rosenbaum, 2002; Rosenbaum, 2002; Rosenbaum and Witman, 2002; Han et al., 2003). Interference with IFT protein function results in loss or reduction of primary cilia (Pazour et al., 2000, 2002a; Pazour and Rosenbaum, 2002). Primary cilia possess cation channels and receptors that appear to activate signal transduction pathways that control cellular function (Pazour and Rosenbaum, 2002; Pazour et al., 2002a; Pazour and Witman, 2003). Polycystin-2 (PC2) is a calcium-selective channel on primary cilia associated with polycystic kidney disease (Somlo and Ehrlich, 2001). It appears to be activated by mechanical movement of primary cilia in response to fluid flow (Nauli et al., 2003), and controls the assembly of primary cilia (Thomson et al., 2003; Watnick et al., 2003). However, little is known about the mechanism by which IFT proteins and PC2 are organized at the centrioles/basal bodies (terms used interchangeably).

A role for centrosome proteins in primary cilia formation has recently been established. Mutants of a *Drosophila* protein that shares homology with the vertebrate centrosome proteins pericentrin (Pcnt) (Flory and Davis, 2003; Zimmerman et al., 2004) and AKAP450 (Keryer et al., 2003) disrupt for-

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Figure 1. **Pcnt localizes to centrioles and basal bodies.** (a) Immunofluorescence image of a centrosome in RPE1 cells costained for Pcnt (green) and centriolin (red; bar, 1 μm). (b and c) DIC (b) and immunofluorescence (c) images of a primary cilium (arrow) in RPE1 cell stained for Pcnt (green) and centrioles/primary cilium (GT335, red). Bar, 5 μm for b and c. (d) Immunofluorescence image of a ciliated epithelial cell from mouse trachea showing Pcnt (green) at the base of motile cilia (DIC; bar, 5 μm). (e) Immunogold electron microscopic image of a ciliated cell (as in d) after incubation with antibodies to Pcnt and secondary antibodies bound to 5-nm gold (bar, 250 nm).
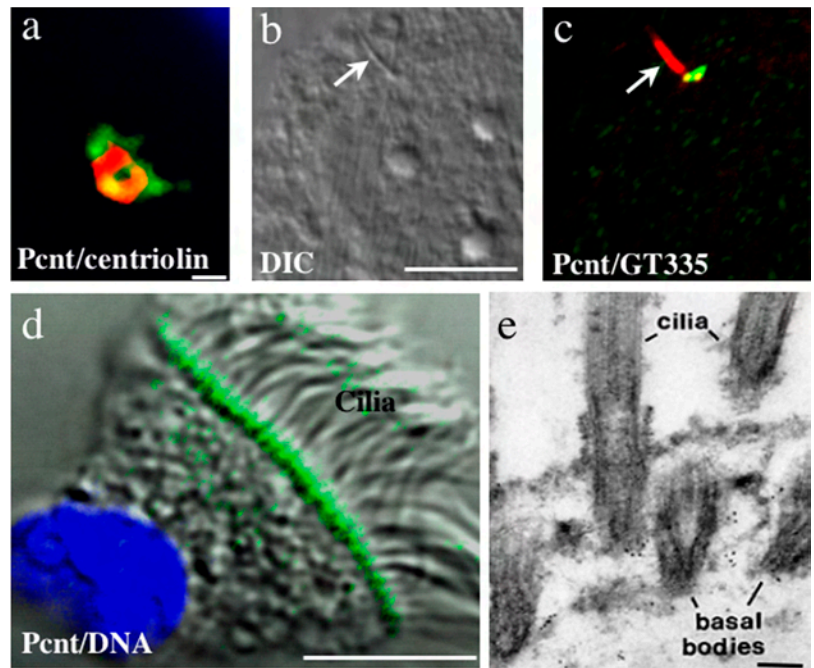
mation of mechanosensory and chemosensory cilia (Martinez-Campos et al., 2004). *Drosophila* mutants that affect IFT also disrupt formation of *Drosophila* sensory cilia (Han et al., 2003). However, the molecular mechanism by which centrosomes and centrosome proteins modulate primary cilia assembly has not been determined. In this report, we show that Pcnt forms a complex with IFT proteins and PC2 in vertebrate cells and tissues, and that Pcnt depletion by small interfering RNAs (siRNAs) disrupts centriole association of IFTs and PC2 and inhibits primary cilia formation.

Results and discussion

In this work, we have studied a larger isoform of Pcnt using specific siRNAs and antibodies unless otherwise noted (Flory and Davis, 2003; Zimmerman et al., 2004). Immunofluorescence imaging demonstrated that Pcnt partially overlapped with centriolin, a protein associated with the mother centriole at centrosomes (Fig. 1 a) (Gromley et al., 2003). In addition, Pcnt associated with both centrioles at the base of primary cilia (Fig. 1, b and c) and motile cilia (Fig. 1 d). Higher resolution immunogold EM demonstrated that Pcnt was on or near the centrioles of motile cilia (Fig. 1 e).

To test the role of Pcnt in cilia organization, we depleted protein levels by siRNA. We observed a 75–90% reduction in protein levels and a dramatic reduction in centrosome levels of Pcnt in most cells (Fig. 2, a–c; arrow in c) when compared with cells treated with control siRNAs targeting lamins A/C (Fig. 2, a and b) or cells that did not respond to siRNA treatment (Fig. 2 c, bottom cell). In contrast, centrosome localization of γ-tubulin was only slightly affected under these conditions (Fig. 2 c, top cell). Primary cilia were induced in retinal pigmented epithelial cells (RPE1) treated with siRNAs targeting Pcnt or lamin A/C. Cilia were detected with antibodies to polyglutamylated tubulins (GT335; Gromley et al., 2003) and by differential interfer-

ence contrast (DIC) microscopy. In most cells treated with siRNAs targeting Pcnt, primary cilia failed to assemble (Fig. 2, e, g, and h), whereas control cells treated with siRNAs targeting lamin or ninein assembled normal full-length primary cilia (Fig. 2, d, f, and h; unpublished data).

To address the mechanism of ciliary loss in cells with reduced Pcnt, we examined centriole function, structure, and composition. Consistent with previous results from our group and others (Dammermann and Merdes, 2002; Martinez-Campos et al., 2004; Zimmerman et al., 2004), we found that microtubule organization and nucleation were not significantly disrupted (unpublished data). In addition, centriole ultrastructure was normal (Fig. 2, i–k; $n = 45$ centrosomes). Centrioles were sometimes separated (Fig. 2, e and g), but this was also observed after functional abrogation of proteins that did not affect primary cilia (e.g., ninein; unpublished data).

Because vertebrate primary cilia formation and function requires IFT proteins (Murcia et al., 2000; Pazour et al., 2000) and the cation channel PC2 (Somlo and Ehrlich, 2001; Pazour et al., 2002b; Rosenbaum and Witman, 2002; Nauli et al., 2003), we reasoned that Pcnt might cooperate with these proteins in primary cilia organization. To test this, we first determined the precise localization of these proteins. IFT57 and IFT88 localized primarily to the distal end of the mother centriole near the base of the primary cilium and to the tips and in spots along the length of primary cilia (Fig. 3, a and b). Localization of these IFTs to the distal portion of the mother centriole was consistent with known sites of IFT protein localization in *Chlamydomonas reinhardtii* (Cole et al., 1998; Deane et al., 2001). IFT20 was found on the proximal portion of mother centriole and the lateral aspect of the daughter centriole (Fig. 3 c), an area thought to be involved in interconnecting the two centrioles. PC2 localized primarily to the mother centriole underlying the primary cilium (Fig. 3 d). In mouse tracheal epithelial cells, IFT proteins partially localized with Pcnt to sites at the base
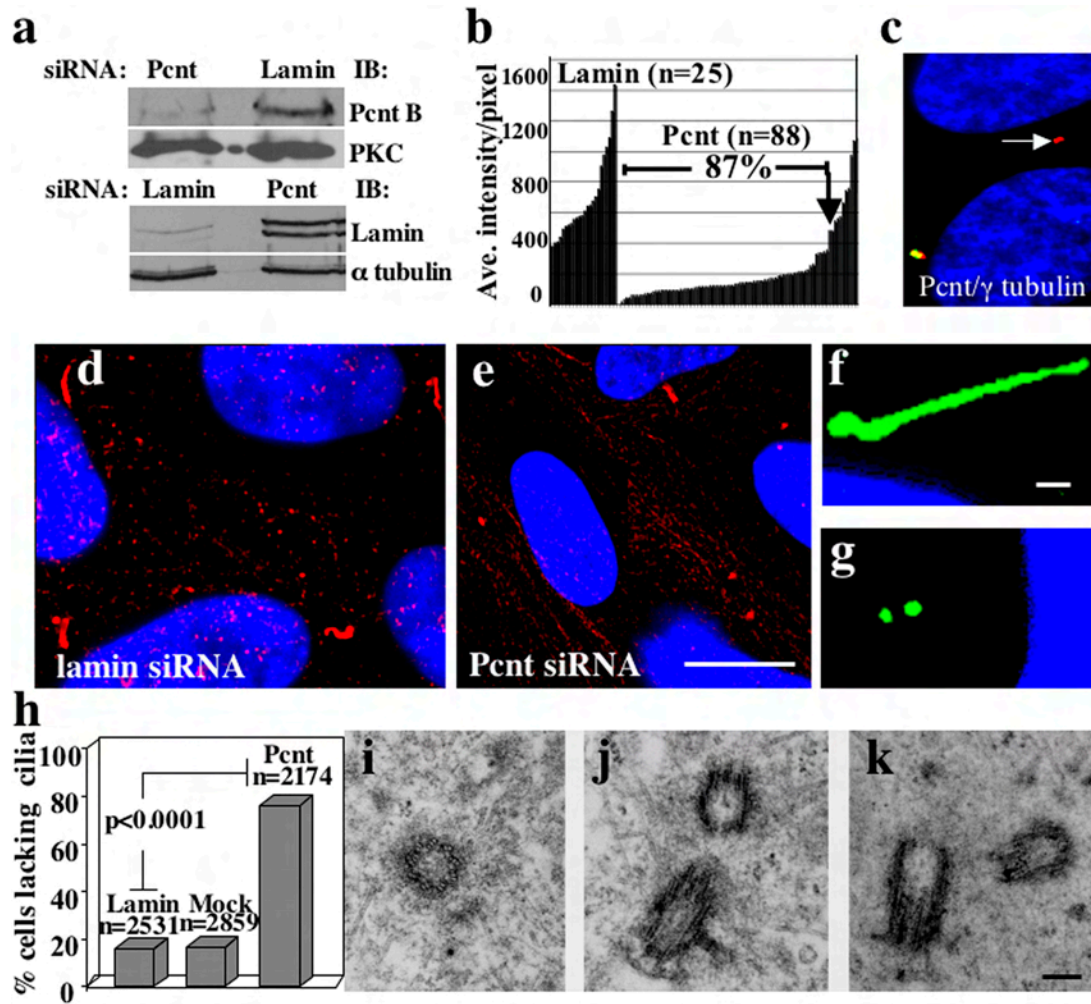
Figure 2. **Pcnt silencing inhibits primary cilia formation.** (a) Pcnt and lamin protein levels (Western blot) after siRNA as indicated. α-Tubulin or PKC, loading controls. (b) Fluorescence intensity of individual centrosomes (bars) after treatment with siRNAs targeting Pcnt or lamin. Centrosomal Pcnt is reduced to levels below the lowest control levels (lamin) in 87% of cells. (c) Immunofluorescence image of RPE1 cells after Pcnt silencing showing reduced centrosomal Pcnt in one cell (green, arrow) and normal level in the other. γ-Tubulin (red) is not significantly affected. Low (d and e) and high (f and g) magnification immunofluorescence images of cilia and centrioles stained with GT335 after treatment with Pcnt (e and g) or lamin (d and f) siRNAs. Bar in e, 5 μm (for d and e); bar in f, 1 μm (for f and g). DNA, blue. (h) Graph showing percentage of cells that lack cilia after treatment with indicated siRNAs. Bars represent average of three experiments. P value, standard *t* test. (i–k) Electron micrographs showing centriole structure in cells with reduced Pcnt. Bar in k, 200 nm (for i–k).

of the motile cilia where basal bodies are found (Fig. 3 e, IFT20).

Next, we addressed the centriolar anchoring mechanism of Pcnt, IFTs, and PC2. We found that Pcnt was dependent on IFT proteins for localization to basal bodies using cells that stably express siRNAs targeting IFT20. These cells showed reduced centriolar IFT20 and lacked primary cilia (Fig. 3, g–g′′ and h) compared with cells of the parent line (Fig. 3, f–f′′ and h). In cells with reduced centriole-associated IFT20, we observed a similar reduction in Pcnt levels (Fig. 3 g, g,′′ and i). In a reciprocal experiment, we found that IFTs and PC2 were dependent on Pcnt for centriole localization. Pcnt localized to both centrioles at the base of cilia, partially colocalized with IFT proteins (Fig. 4 a′′, IFT57) and totally overlapped with PC2 (Fig. 4 c′′). Pcnt silencing reduced the levels of centriolar Pcnt (Fig. 4 b, b′′, c, and c′′; top cell), IFT57 (Fig. 4, b′–b′′), IFT20, IFT88 (unpublished data), and PC2 (Fig. 4, c′–c′′; top cell). In contrast, adjacent nontransfected cells

or cells treated with lamin siRNAs had robust staining for IFT57 and PC2 (Fig. 4, a′–a′′ and c′–c′′; bottom cell). These results show that Pcnt and IFTs are codependent in their localization to basal bodies of primary cilia.

Previous reports showed that IFT protein complexes and Pcnt complexes had similar S values on sucrose gradients (17–18S; Dictenberg et al., 1998; Pazour et al., 2002a). To determine whether Pcnt interacted with IFT proteins, we isolated IFT complexes by a multistep procedure (San Agustin and Witman, 2001) and found that Pcnt A and B cofractionated with IFT88 in the final gel filtration column (Fig. 5 a). Based on recent data showing that IFT71 is present on centrosomes and spindle poles (Iomini et al., 2004), we analyzed centrosome preparations (Doxsey et al., 1994) for the presence of IFT proteins. IFT88 (Fig. 5 b) and other IFT proteins (unpublished data) were present in pooled fractions containing centrosome proteins (γ-tubulin, Pcnt), but not in pooled fractions lacking centrosomes. Moreover, our immu-
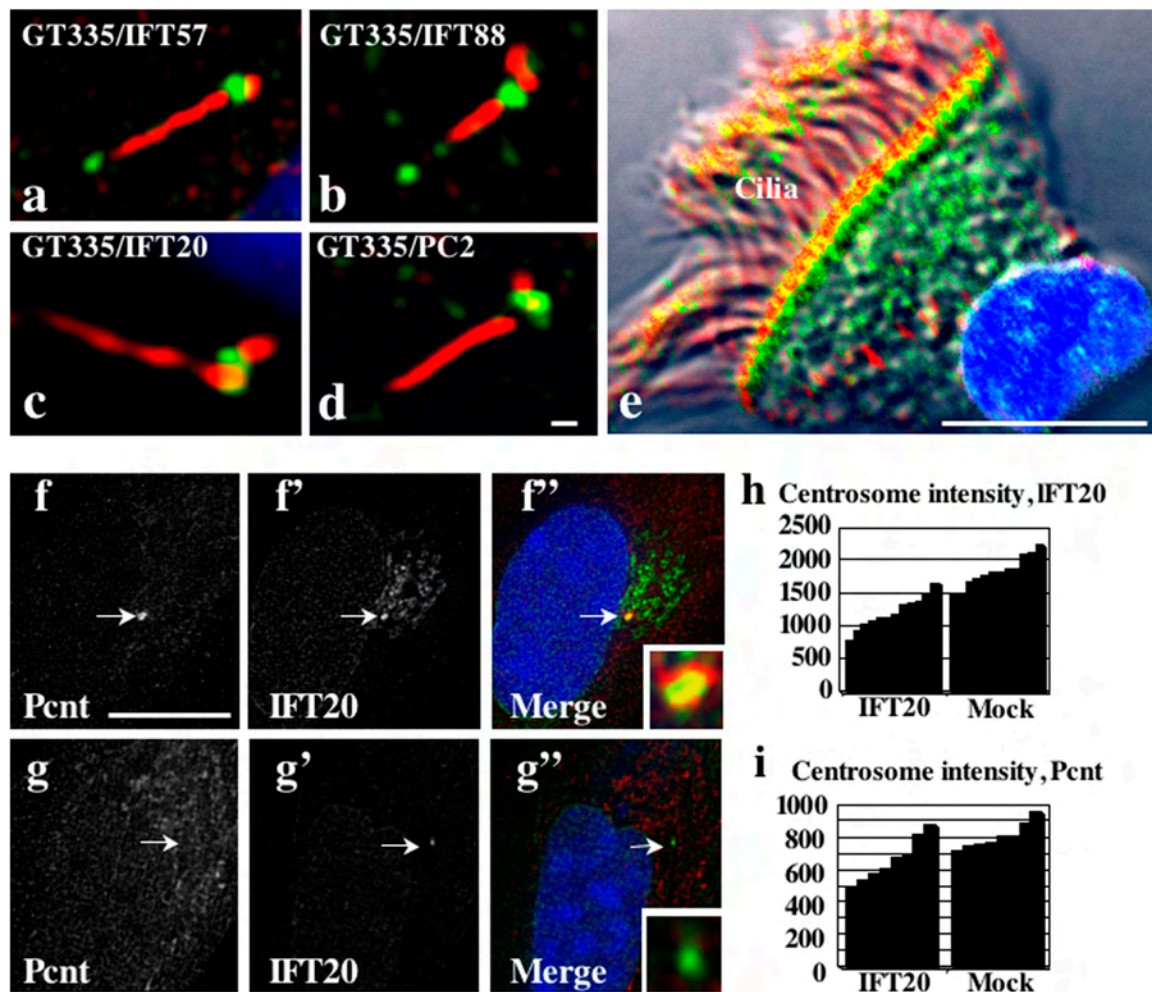
Figure 3. **Localization of IFT proteins and PC2, and mislocalization of Pcnt in cells with reduced IFT20.** (a–d) RPE1 cells stained for IFT57, IFT88, IFT20, and PC2 (green) and for basal bodies/cilia (GT335, red). Bar in d, 1 μm. (e) Pcnt (green) partially colocalizes with IFT20 (red) at the base of motile cilia (seen by DIC) in mouse epithelial cells. DNA, blue. Bar, 5 μm. (f–g'') Untreated RPE1 cells (f–f'') or RPE1 cells stably expressing siRNA targeting IFT20 (g–g'') showing centrosomal levels of IFT20 (f' and g'), Pcnt (f and g; bar, 5 μm), or merge (f'' and g''). Pcnt, red, IFT20, green, DNA, blue at arrows. Insets, enlargements of f'' and g''. (h and i) Fluorescence intensity of IFT20 (h) and Pcnt (i) at individual centrosomes (bars) in cells stably expressing IFT20 siRNA or mock, as indicated below graph.

nofluorescence imaging showed that IFT20, 57, 88, and PC2 were abundant at centrosomes in interphase cells and spindle poles during mitosis in RPE1 cells (unpublished data).

Immunoprecipitation of Pcnt using antibodies (that recognize both small, Pcnt A and large, Pcnt B isoforms) raised to two independent domains pulled down endogenous IFT88 from two ciliated cell lines (Fig. 5 c, two top panels); endogenous IFT57 from testes and ciliated cells (unpublished data), and ectopically expressed GST-GFP-IFT20 (Fig. 5 c, bottom) and endogenous PC2 from mitotic cells (Fig. 5 d). We observed no coimmunoprecipitation of any IFT protein or PC2 when Pcnt antibody was omitted (Fig. 5 d; Bd) or substituted with a nonimmune IgG (Fig. 5, c and e; IgG). In reciprocal experiments we found that PC2 immunoprecipitation pulled down endogenous IFT57 from ciliated cells (Fig. 5 e) and that IFT57 pulled down IFT88 (Fig. 5 c). Together, these biochemical data suggest that Pcnt, PC2, and IFT proteins form a complex in the cytoplasm of vertebrate cells.

The data in this manuscript show that Pcnt binds IFT proteins and PC2 and is required for primary cilia formation in

human cells. This suggests a model in which Pcnt recruits protein complexes involved in cilia assembly and calcium signaling to centrioles at the base of primary cilia (and perhaps flagella). Because *Drosophila* Pcnt/AKAP450 and IFT were shown separately to function in primary cilia assembly (Han et al., 2003; Martinez-Campos et al., 2004), it is possible that Pcnt has a conserved function in IFT organization during cilia formation in both *Drosophila* and vertebrate cells.

IFT does not appear to play a role in assembly or function of *Drosophila* sperm flagella (Han et al., 2003) as seen in other organisms (Rosenbaum and Witman, 2002). Thus, it is unlikely that defects in flagellar motility in *Drosophila* Pcnt/AKAP450 mutants (Martinez-Campos et al., 2004) are a consequence of disruption of the Pcnt–IFT interaction. However, both vertebrate Pcnt (Dictenberg et al., 1998; Takahashi et al., 2002; Zimmerman et al., 2004) and *Drosophila* Pcnt/AKAP450 (Kawaguchi and Zheng, 2003) interact with complexes containing γ-tubulin, and γ-tubulin has recently been shown to be required for flagellar motility in trypanosomes (McKean et al., 2003). Thus, it is possible that disrup-
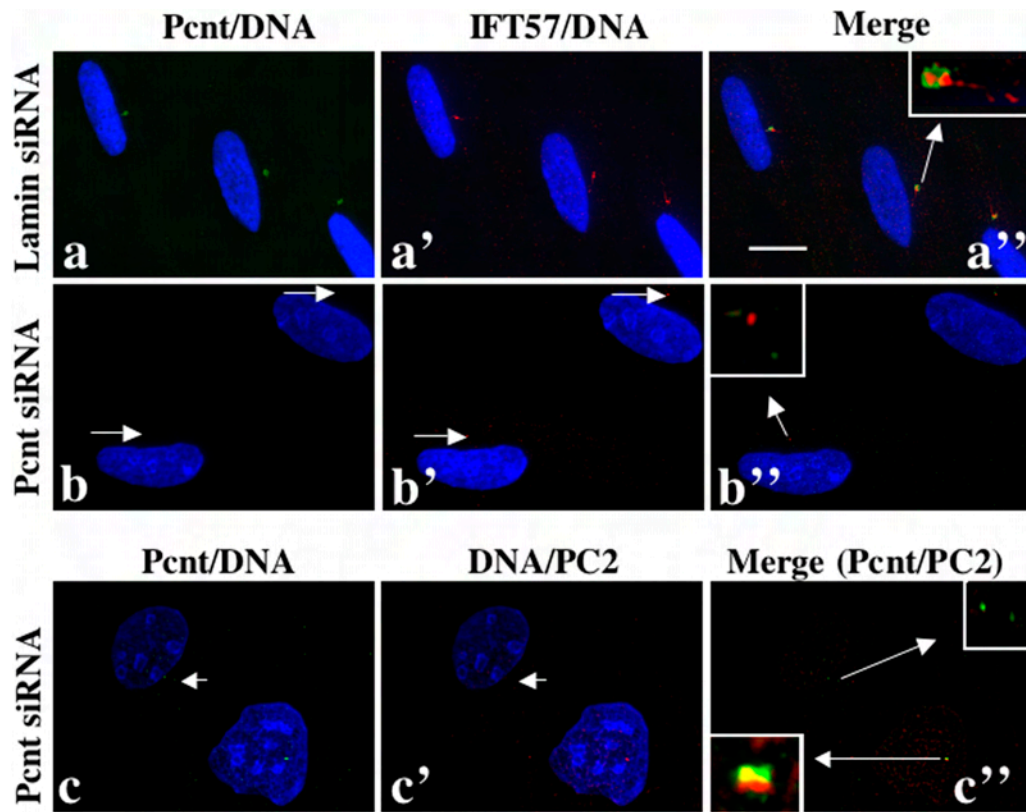
Figure 4. **Pcnt colocalizes to basal bodies with IFT proteins and PC2, and Pcnt silencing mislocalizes IFT proteins and PC2 from basal bodies and centrosomes.** (a–c'') IFT57 (a–b'' red) and PC2 (c–c'', red) are mislocalized from basal bodies in RPE1 cells with reduced Pcnt (b–b'', arrows; c–c'', green, small arrows), but not in RPE1 cells treated with lamin siRNAs (a–a''; bar, 10 μm) or in the cell with control level of Pcnt (c and c'', bottom). Insets: higher magnification of a'', b'', and c'' as indicated by arrows. DNA, blue.

tion of the interaction between Pcnt/AKAP450 and γ-tubulin complexes could account for lack of motility in *Drosophila* flagella. Another possibility is that the observed structural alterations in centrioles in spermatocytes from *Drosophila* Pcnt/AKAP450 mutants (Martinez-Campos et al., 2004) could contribute to defects in both cilia and flagella. However, in this work we did not detect changes in centriole structure in cells depleted of Pcnt. Given the recent findings that Pcnt and other centrosome proteins are integral components of cilia and flagella and that IFT proteins and PC2 are integral components of centrosomes and spindle poles (Iomini et al., 2004; unpublished data), it is likely that perturbation of proteins in one of these compartments affects the function of the other. Because defects in centrosomes and spindle poles are well documented in cells with abrogated Pcnt and Pcnt orthologues, they could also contribute to defects in centrosome derivatives such as cilia and flagella. Moreover, Pcnt and IFT proteins require molecular motors to mediate transport events, so it is possible that they utilize some of the same components to accomplish these functions (Zimmerman and Doxsey, 2000). It is clear from this discussion that a better understanding of the precise role of Pcnt in cilia and flagella assembly/function will require additional studies.

On a final note, it is interesting that centrosomes in *Drosophila* Pcnt/AKAP450 mutants are disorganized but appear to assemble normal mitotic spindles (Martinez-Campos et al., 2004). It is possible that residual functional protein remaining in *Drosophila* mutants is sufficient for spindle func-

tion. However, recent results from vertebrate cells indicate that there are several forms of Pcnt (Flory and Davis, 2003), and that a smaller form of the protein is required for spindle organization and function, possibly through its role in anchoring γ-tubulin complexes or IFT proteins at spindle poles (Zimmerman et al., 2004). A larger Pcnt isoform that shares homology with *Drosophila* Pcnt/AKAP450 does not have a dramatic effect on spindle organization (Zimmerman et al., 2004). It is likely that the multiple Pcnt isoforms contribute to a multitude of cellular functions.

Materials and methods

Cells, siRNAs, IFT isolation, and primary cilia formation
Cells used in this work, RPE1 (Morales et al., 1999), a mouse inner medullary collecting duct (IMCD3), primary cells isolated from Tg737 wild-type mouse (488) (Pazour et al., 2000), and freshly isolated primary mouse trachea cells were grown as described in American Type Culture Collection. Trachea dissected from mice in PBS were opened and scraped with a wooden applicator stick. Released ciliated epithelial cells were spun onto coverslips and fixed in −20°C methanol. siRNAs (21-nt; Dharmacon Research, Inc.) targeting Pcnt B (GenBank/EMBL/DDBJ accession no. XM_036857; nt 301–319), Pcnt A/B, or ninein (Dammermann and Merdes, 2002) and lamin A/C (Gromley et al., 2003) were delivered to cells at 200 nM (Oligofectamine; Invitrogen). We also used a stable RPE1 cell line expressing IFT20-specific siRNAs (5'-GGAAGAGTGCAAAGA-CTTT-3'; Follit and Pazour, in preparation). IFT protein fractions were prepared as described previously (San Agustin and Witman, 2001) using additional protease A inhibitors (Complete Mini tablets; Roche) in lysis buffer. Primary cilia were induced after siRNA treatment (72 h) by culturing RPE1 cells in medium with 0.25% serum and siRNAs for 48 h and were identified using GT335 antibody and DIC microscopy.

Figure 5. **Pcnt interacts with proteins involved in cilia assembly and function.** (a) Pooled IFT fractions from a sucrose gradient from mouse testes were applied to an FPLC column and fractions were loaded on SDS gels and probed with Pcnt antibodies or IFT88 antibody. (b) Pooled peak centrosome fractions from sucrose gradients (+) containing γ-tubulin, Pcnt, and IFT88 as indicated and pooled noncentrosome fractions (−). (c, top) Pcnt NH$_2$- and COOH-terminal antibodies (PcN, PcC) independently immunoprecipitated endogenous IFT88 from lysates of ciliated 488 cells. IgG, nonimmune rabbit IgG, lysates (Lys) showing IFT88 at right. Pcnt immuno-precipitation confirmed (right). (c, middle) PcC/N immunoprecipitated IFT88 from ciliated RPE1 cells, as did antibodies to IFT88 and IFT57 but not rabbit IgG. (c, bottom) PcN and PcC pull down a GST–GFP–IFT20 fusion protein from a cell line stably overexpressing the protein, as does a glutathione column (IFT20), but not nonimmune IgG. Blots were probed with anti-GFP antibodies, immunoprecipitation with Pcnt (right); IB, immunoblot antibody. (d) PcN immunoprecipitated PC2 from mitotic RPE1 cells, whereas beads alone did not (Bd). Pcnt immunoprecipitation confirmed by immunoblot (Pcnt B). (e) PC2 antibody, but not rabbit IgG, immunoprecipitated IFT57 from ciliated 488 cells. IFT57, top band. Antibody heavy chain, bottom band.
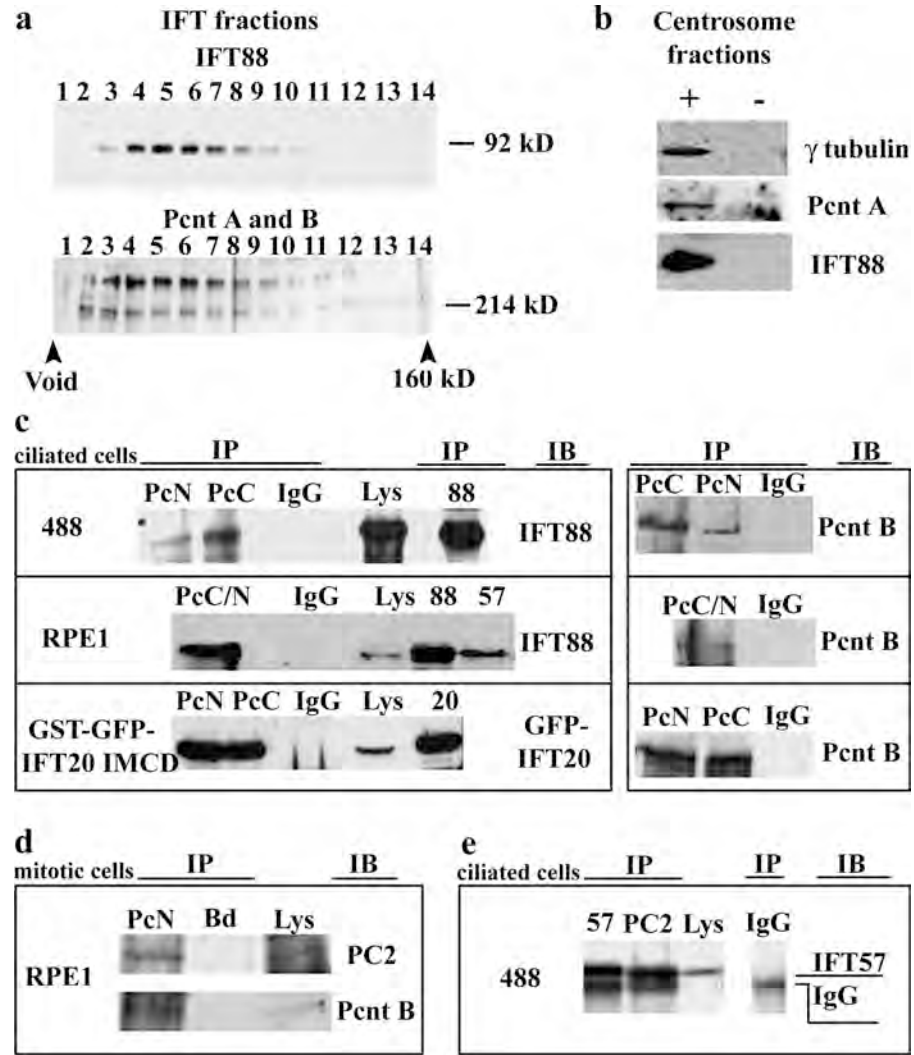
Immunofluorescence, EM, and RT-PCR

Cells were prepared for immunofluorescence, imaged, deconvolved (Meta-Morph; Universal Imaging Corp.), and displayed as two-dimensional projections of three-dimensional reconstructions to visualize the entire cell volume as described in Gromley et al. (2003). We used methanol as fixative, then confirmed using formaldehyde fixation as previously shown (Dictenberg et al., 1998). Immunogold EM was performed as described previously (Doxsey et al., 1994). RT-PCR for amplification of Pcnt B (forward primer 5′-AACACTCTCCATGATTGCCC-3′ and reverse 5′-TAC-CCTCCCAATCTTTGCTG-3′) and α-tubulin was performed as described previously (Gromley et al., 2003).

Immunoprecipitation, Western blotting, and antibodies

Cells were lysed in lysis buffer consisting of 50 mM Tris-HCl (pH 7.5), 10 mM Na$_2$HPO$_4$ (pH 7.2), 1 mM EDTA, 150 mM NaCl, 1% IGEPAL CA-1630, and Complete Mini tablets. Testes lysates were prepared as described previously (San Agustin and Witman, 2001). Antibodies were added to freshly prepared cell extracts and were incubated at 4°C overnight. Protein A/G Plus-Agarose (Santa Cruz Biotechnology, Inc.) or Glutathione Sepharose 4B (Amersham Biosciences) was washed in lysis buffer, added to the cell extracts, and incubated for 2 h at 4°C. The beads were washed and resuspended in sample buffer. 5% SDS-PAGE gels were used to detect Pcnt and PC2, and 10% gels to detect IFTs. Controls included cell extracts incubated with rabbit IgG or beads alone. No bands were seen with control IgGs under any of these conditions or when control IgGs were used at concentrations >10-fold higher than experimental samples. Cell extracts used in this work for the Pcnt IFT interactions came from cells grown in 0.25% serum for 48 h to induce cilia formation. We used affinity-purified antibodies against the NH$_2$ and COOH termini of Pcnt A/B (PcN,

PcC; Doxsey et al., 1994; Dictenberg et al., 1998), Pcnt B (a gift of T. Davis, University of Washington, Seattle, WA; Flory et al., 2000), IFT proteins (Pazour et al., 2002a), PC2 (Scheffers et al., 2002), centriolin (Gromley et al., 2003), GT335 (a gift of P. Denoulet, Université Pierre et Marie Curie, Paris, France; Wolff et al., 1992), α-tubulin (Sigma-Aldrich), and lamin A/C (Cell Signaling Technology).

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Loss of centrosome integrity induces p38–p53–p21-dependent G1–S arrest

Keith Mikule[1,4,5], Benedicte Delaval[1,5], Philipp Kaldis[2], Agata Jurcyzk[1], Polla Hergert[3] and Stephen Doxsey[1,6]

Centrosomes organize the microtubule cytoskeleton for both interphase and mitotic functions. They are implicated in cell-cycle progression but the mechanism is unknown. Here, we show that depletion of 14 out of 15 centrosome proteins arrests human diploid cells in G1 with reduced Cdk2–cyclin A activity and that expression of a centrosome-disrupting dominant-negative construct gives similar results. Cell-cycle arrest is always accompanied by defects in centrosome structure and function (for example, duplication and primary cilia assembly). The arrest occurs from within G1, excluding contributions from mitosis and cytokinesis. The arrest requires p38, p53 and p21, and is preceded by p38-dependent activation and centrosomal recruitment of p53. p53-deficient cells fail to arrest, leading to centrosome and spindle dysfunction and aneuploidy. We propose that loss of centrosome integrity activates a checkpoint that inhibits G1–S progression. This model satisfies the definition of a checkpoint in having three elements: a perturbation that is sensed, a transducer (p53) and a receiver (p21).

The centrosome is primarily known for its microtubule organizing function. However, centrosomes contain hundreds of proteins with diverse functions suggesting roles in numerous cellular activities[1]. Animal cell centrosomes are structurally complex organelles comprised of two microtubule–based centrioles surrounded by a protein matrix (pericentriolar material, PCM) and other structural elements. Similarly to DNA replication, the centrosome duplication process occurs once per cell cycle, is semi-conservative, initiates in G1 and is controlled by the same cyclin-dependent kinases[2,3]. A single interphase centrosome yields two mature centrosomes at mitosis, which participate in organization of bipolar spindles and segregation of chromosomes. Most human carcinomas are characterized by aberrant centrosomes[4,5], which are thought to organize dysfunctional spindles and contribute to genetic instability.

Recent studies indicate that centrosomes have a role in cytokinesis and that disruption of this function is associated with cell-cycle arrest. For example, when centrosomes are removed[6,7] or disrutped[8], cytokinesis is impaired and cells arrest in G1. In contrast, cell-cycle progression is not affected in cells with extra centrosomes, extra nuclei or after pharmacological disruption of cytokinesis[9,10]. These results indicate that centrosome-associated G1 arrest occurs only when centrosomes are absent or compromised. Other studies demonstrate that progression from G1 into S phase requires binding of cell-cycle regulatory molecules to centrosomes[1,11]. However, little is known about how centrosomes contribute to the G1 to S phase transition.

RESULTS

Depletion of centrosome proteins reduces their centrosome levels and induces G1 arrest

To address the role of centrosomes in cell-cycle progression, we targeted fifteen centrosome proteins for depletion using small-interfering RNAs (siRNAs). We used human diploid epithelial cells (RPE-1) and confirmed results with three other human diploid cell lines (see Methods). Targeted proteins included integral centrosome and/or centriole components, as well as regulatory proteins that affect centrosome function. Three non-centrosomal proteins served as negative controls (including the intermediate filament protein lamin and the actin-associated protein zyxin). Indirect immunofluorescence staining of individual cells revealed a consistent reduction in the centrosome-associated fraction of all targeted centrosome proteins (Fig. 1a and see Supplementary Information, Fig. S1a–c), even though many have significant cytoplasmic fractions. Western blots of whole-cell lysates showed reduction in the global level of targeted proteins (see Supplementary Information, Figs S1d).

Depletion of 14–15 centrosome proteins induced G1 arrest (Fig. 1). Cells failed to reach confluency, exhibited a low mitotic index, did not progress into S phase (BrdU-negative, Fig. 1b) and showed reduced reactivity for the proliferating antigen Ki-67 (Fig. 1c)[12]. Flow cytometry analysis of pericentrin-depleted cells revealed a small increase in the G1 peak (2N DNA content) over controls (Fig. 1d) and a concomitant decrease in the G2 peak was observed. Similar changes were observed for depletion of the centrosome proteins centriolin and PCM1, but not
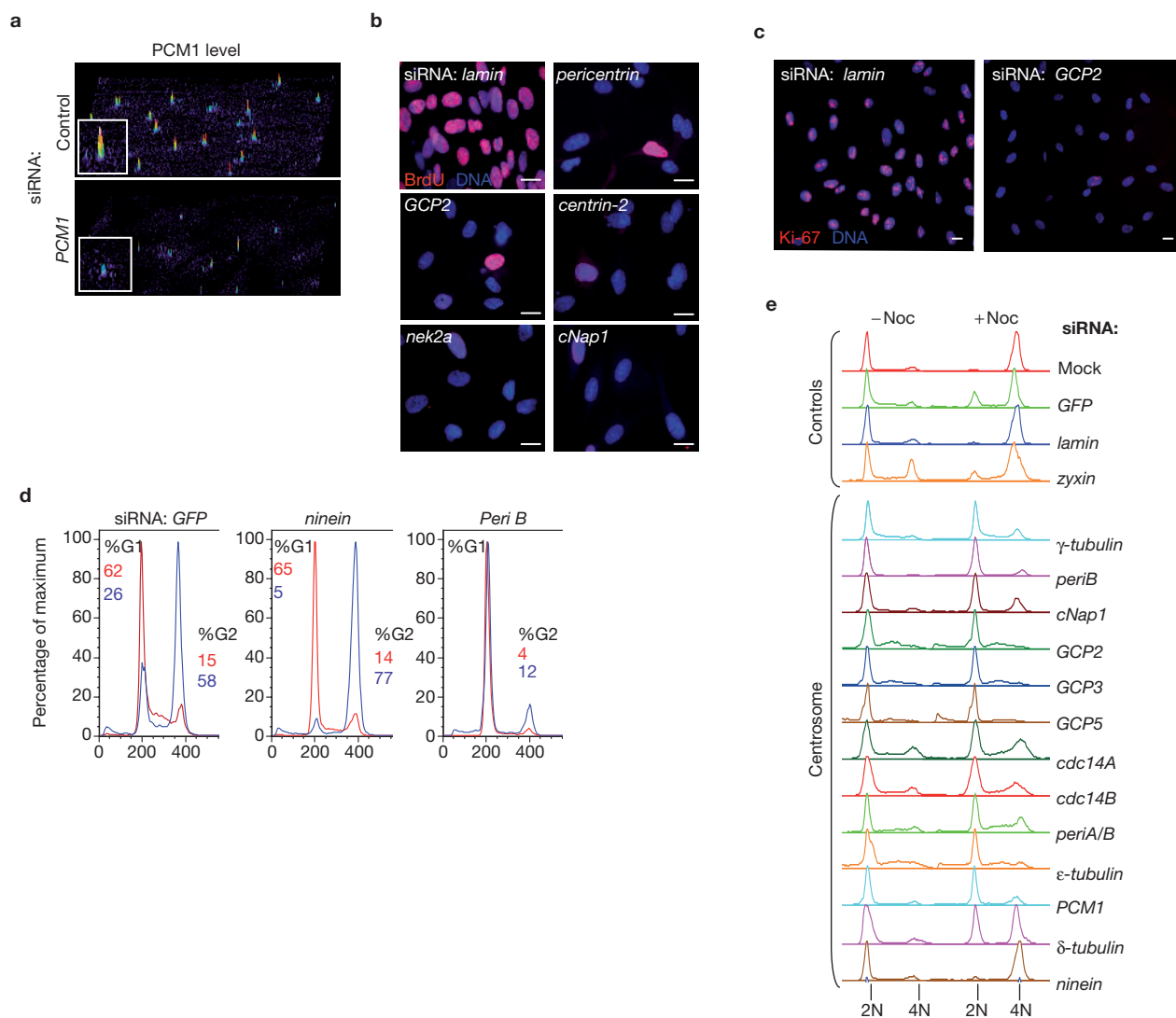
Figure 1 siRNA-mediated centrosome protein depletion triggers G1 arrest. (**a**) Semi-quantitative pixel intensity profiles of centrosomes produced from optically sectioned (*z*-axis) fluorescence images show depletion of centrosomal PCM1 (inset shows enlarged pixel intensity profile of centrosome). Colour indicates staining intensity. Profiles for other proteins are shown in the Supplementary Information, Fig. S1. (**b**) BrdU incorporation (16 h pulse) in siRNA-treated (72 h) cells, as indicated. (**c**) Immunoflorescence microscopy images showing that Ki-67 staining is not present in nuclei of most *GCP2* siRNA-treated cells (72 h) but is present in cycling control cells (*lamin*). (**d**) Flow cytometry profiles of cells labelled with propidium iodide. Cells were treated with indicated siRNAs for 72 h without (red, DMSO) and with (blue) nocodazole for the final 12 h (>10000 cells per profile). More profiles are shown in the Supplementary Information, Fig. S2a. (**e**) Flow cytometry profiles of cells treated with the indicated siRNAs for 72 h then with DMSO (– noc) or nocodazole (+ noc) for the final 12 h. Profiles are representative of three experiments (>5000 cells per profile). The scale bar represents 10 μm in **b** and **c**.

for ninein (data not shown, Fig. 1d). To highlight cell-cycle differences, cells were treated with nocodazole to depolymerize microtubules, activate the spindle assembly checkpoint and accumulate cells in G2–M with 4N DNA content. Remarkably, nearly all centrosome-depleted cells were retained in the G1 peak with 2N DNA content and did not shift to the G2–M peak, whereas control cells did (Fig. 1d, e). Depletion of all centrosome proteins but one induced G1 arrest (Fig. 1b, e). Cells treated with siRNAs targeting control RNAs or *ninein*[13] continued to cycle normally. *Ninein* depletion had no effect on cell-cycle progression despite the fact that it was significantly reduced at centrosomes following treatment with two independent siRNAs (see Supplementary Information, Fig. S1c). We did not determine whether the lack of cell-cycle arrest in

ninein-depleted cells (and the partial arrest in *δ-tubulin*-depleted cells, Fig. 1e) was due to insufficient protein reduction, a lack of function in this pathway or the presence of proteins with redundant functions (for example, ninein-like protein[14]). Targeted centrosome proteins were localized to different centrosomal sites including PCM, centrioles, mother centriole or subdistal appendages, suggesting that a single centrosome substructure was not involved in the arrest.

G1 arrest is specifically suppressed by re-expression of the target protein

In addition to several control RNAs targeted in our siRNA experiments, we performed other experiments to demonstrate that the cell-cycle arrest
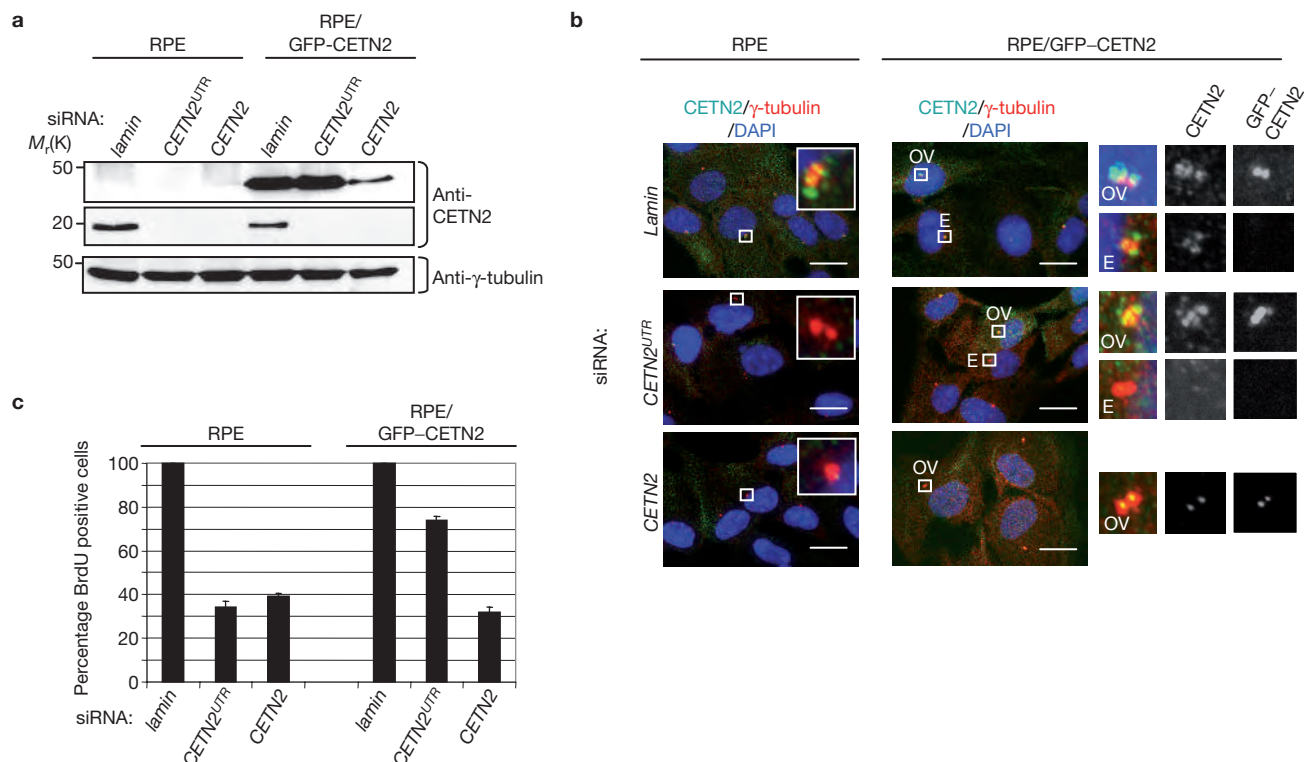
Figure 2 G1 arrest can specifically be suppressed by overexpression of the target protein. (**a**) Western blots from RPE and RPE/ GFP–Cetn2 cells treated with siRNAs as indicated and probed with centrin antibody (20H5) to show endogenous (middle panel) and overexpressed (upper panel) centrin. Lamin, control. *Cetn2*[UTR] siRNA targets only endogenous *Cetn2*, *Cetn2* siRNA targets both endogenous *Cetn2* and GFP–*Cetn2*. γ-tubulin staining demonstrates equivalent loading conditions. (**b**) Immunofluorescence microscopy images taken from siRNA-treated RPE cells (72 h) show reduced level of endogenous Cetn2 protein (green) at the centrosome with both *Cetn2*[UTR] siRNA and *Cetn2* siRNA, when compared with normal levels obtained with

lamin siRNA (insets show enlargements of indicated centrosomes). Images taken from siRNA-treated RPE overexpressing GFP–Cetn2 (right columns) show reduced level of overexpressed GFP-Cetn2 at the centrosome with *Cetn2* siRNA but not with *Cetn2*[UTR] siRNA. Enlargements of centrosomes from an overexpressing cell (ov, top inset from each picture) and a non overexpressing cell in the same field (E, endogenous, bottom inset) are shown on the right. γ-tubulin, centrosome marker (red). The scale bar represents 10 μm. (**c**) Quantification of BrdU incorporation (16 h pulse) in siRNA-treated RPE and RPE cells overerepssing GFP-Cetn2. Average of three independent experiments ± s.e.m. is shown.

was specific for depletion of centrosome proteins. We first tested whether G1 arrest could be suppressed by re-expression of the targeted RNA. An siRNA that targeted the 5′-untranslated region (UTR) of endogenous *centrin 2*, but did not target ectopically expressed GFP–centrin 2, was used (Fig. 2). As expected, the UTR-directed siRNA efficiently depleted endogenous *centrin 2* but not GFP–centrin 2, as determined by immunoblotting and immunofluorescence microscopy (Fig. 2a, b). Cell-cycle analysis demonstrated that control cells depleted of endogenous *centrin 2* arrested, whereas most cells ectopically expressing GFP–centrin 2 continued to cycle (Fig. 2c). When GFP–centrin 2-expressing cells were treated with a siRNA targeting both endogenous *centrin 2* and *GFP–centrin 2* (Fig.2b), G1 arrest was induced (Fig. 2a–c). This demonstrated that centrin 2 was specifically required for cell-cycle progression and suggested that centrosome localization was important for this function (GFP-tagged centrin localized to centrosomes, data not shown).

In a separate experiment, cells were kept under sustained cell-cycle arrest (for 10 days) by repeated treatment with siRNAs targeting *pericentrin* (see Supplementary Information, Fig. S2b). When siRNAs were washed out, cells resumed cycling only after pericentrin was re-expressed and localized properly to centrosomes. These two 'rescue' experiments demonstrate that centrosome protein depletion induces a specific and reversible G1 arrest.

Mislocalization of centrosomal pericentrin by a dominant-negative pericentrin domain induces G1 arrest

To more specifically test whether loss of protein from centrosomes induced G1 arrest, we expressed the carboxyl-terminus of pericentrin (Peri[CT]), which functions in a dominant-negative manner by disrupting the centrosome-bound fraction of endogenous pericentrin[15]. Most cells expressing RFP-tagged Peri[CT] were unable to incorporate BrdU after a 24-h pulse, whereas those expressing RFP alone were mostly BrdU-positive (Fig. 3a, 17% versus 98% BrdU+, *n* = 50 cells). This experiment demonstrates that cell-cycle arrest can be induced by ectopic expression of a centrosome protein, in addition to centrosome protein depletion.

Other studies have shown that a G1-like arrest can be induced by microinjection of PCM1 antibodies into mouse embryos[16] or centriolin antibodies into *Xenopus* embryos[8]. Thus, G1 arrest can be induced by centrosome protein depletion, overexpression or antibody binding in both cultured cells and multicellular organisms. Cell-cycle arrest by three independent centrosome-targeting methods in three different experimental systems argues that this phenomenon is specific for centrosomes.

G1 arrest can be induced in postmitotic cells from within G1

We next examined whether G1 arrest was a consequence of mitotic defects. In the experiment shown in Fig. 3a, cells were identified by time-lapse
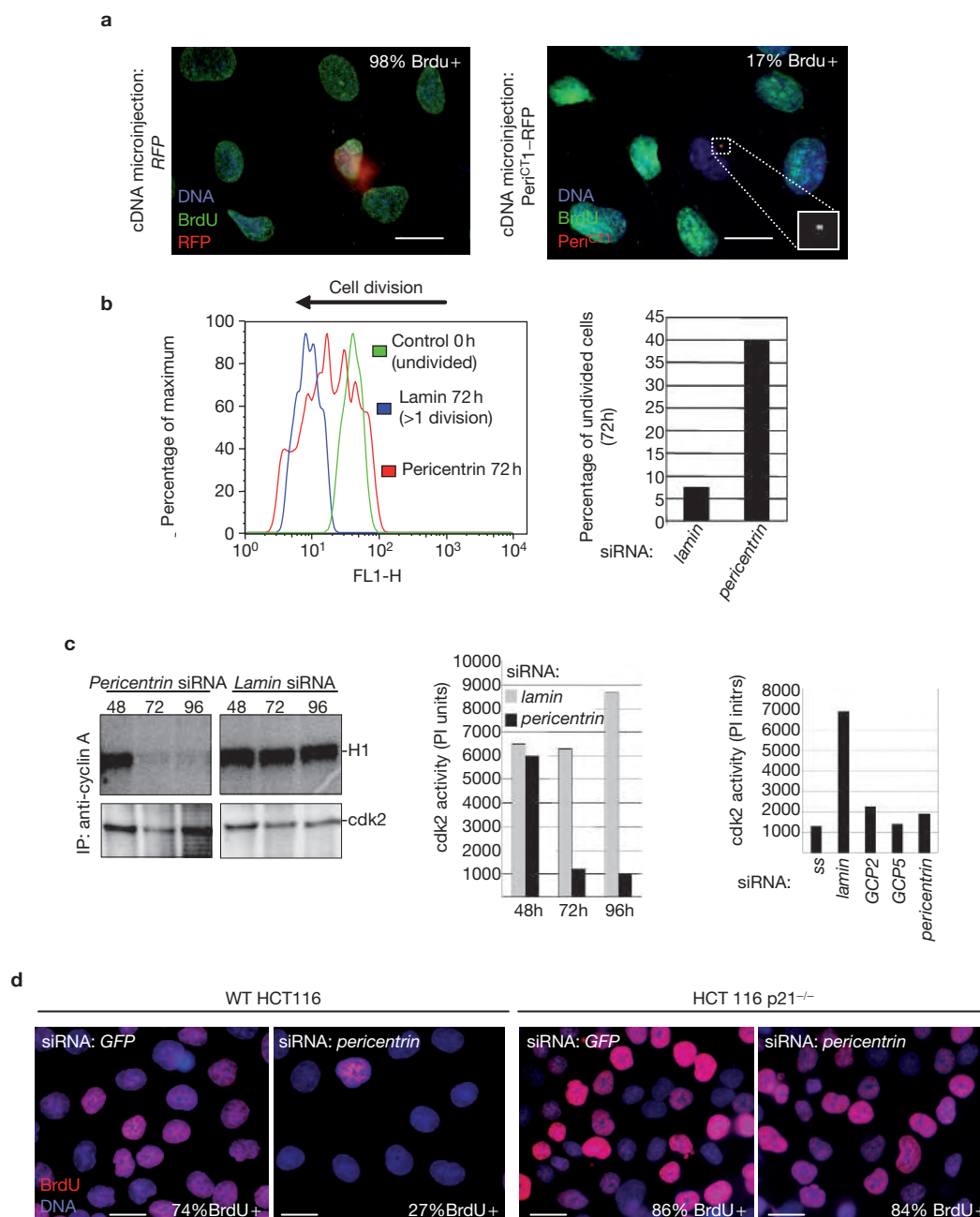
Figure 3 G1 arrest can be induced from within G1, occurs in late G1 with reduced Cdk2–cyclin A activity and is suppressed by deletion of the *p21* gene. (**a**) Cells microinjected in late telophase or early G$_1$ with plasmids encoding either the Peri^{CT}–RFP or RFP alone, as indicated. BrdU incorporation was determined 24 h later (inset shows enlargement of the centrosome of a Peri^{CT}–RFP expressing cell with no BrdU incorporation, nonexpressing cells in the same field incorporate BrdU). Quantification shows that most Peri^{CT}–RFP expressing cells do not incorporate BrdU. (**b**) Cells accumulated in G1–G0 with low serum were loaded with a fluorescent probe to track population doublings then treated with the indicated siRNAs to deplete proteins within G1. Flow cytometry was performed on one set of samples before addition of siRNA to serve as a nondivided control population (green trace). Other samples were treated with indicated siRNAs for 72 h. Serum was added during the last 24 h to induce cycling. Flow cytometry profiles show that a population of *pericentrin* siRNA-treated cells (arrested by serum starvation at the time of transfection) retains the original label showing that cells did not divide at all (left, red trace within green) and others seemed to arrest after one or two divisions (additional peaks due to the fact that 24 h after serum starvation some cells are not yet arrested). Few *lamin*-depleted control cells were found in the undivided peak; most divided two or three times (blue trace). The histogram shows percentage of cells that did not divide. All results are representative of three experiments. (**c**) Cyclin A immunoprecipitations and cdk2 immunoprecipitation–kinase assays from cells treated with indicated siRNAs. Autoradiographs of histone H1 phosphorylation from cyclin A immunoprecipitations and immunoblots for cdk2 are shown. Results representative of three experiments. The histograms show quantification of histone H1 phosphorylation by cdk2 after normalizing for cdk2 levels. PI, phosphorimager units; ss, serum starved 0.25% serum. (**d**) BrdU incorporation (16 h pulse) in HCT116 and HCT116 *p21*$^{-/-}$ cells as indicated following 60 h siRNA-treatment as indicated. The scale bars represent 10 µm in **a** and **d**.
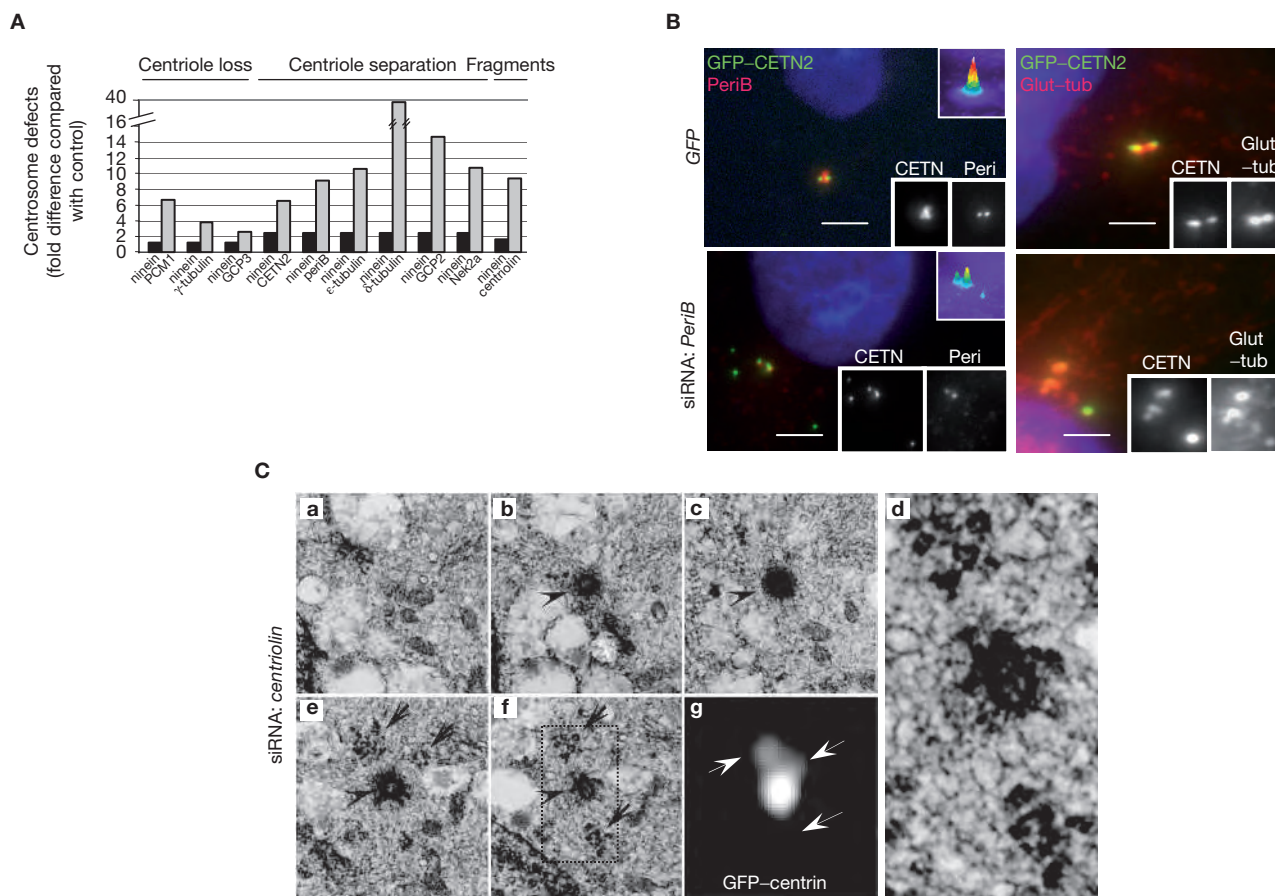
Figure 4 G1 arrested cells show defects in centrosome structure and organization. (**A**) Quantification of cells with defects in centrosome structure and organization following siRNA treatment for the indicated proteins (fold difference normalized to GFP and compared to ninein, see Methods). The results are representative of two experiments (see Supplementary Information, Fig. S3). (**B**) Immunofluorescence microscopy images of seemingly incomplete centrosome-like structures that stain for centriole markers (centrin, CETN and polyglutamylated tubulin; Glut-tub) but not for PCM markers (pericentrin, peri; or γ-tubulin) in RPE-1 cells stably expressing GFP–centrin2 and depleted of the indicated centrosome proteins. The scale bars represent 2 μm. (**C**) Correlative electron microscopy of incomplete centrosome-like structures in serial sections (**a**–**e**) observed by immunofluorescence microscopy (**d**, arrows) and the two parent centrioles (arrowheads indicate daughter centriole in **b** and **c** and mother centriole in **d** and **e**). Enlargement of box in **e** shows mother centriole (**g**, centre) and incomplete centrosome-like structures (above and below).

imaging as they completed cytokinesis[17], and were then microinjected with a plasmid containing the dominant-negative pericentrin construct to induce protein expression in G1. As described earlier, G1-stage pericentrin-expressing cells never entered S-phase, whereas control cells continued to cycle, demonstrating that arrest was induced in postmitotic cells (Fig. 3a). In a second strategy, cells were accumulated in G0–G1 by serum withdrawal then treated with siRNAs to deplete *pericentrin*. Subsequently, bulk cellular proteins were labelled with a fluorescent dye (CFDA-SE), the total amount of which is halved after each cell division[18], and serum was added to release cells from the G0–G1 arrest. Nearly all control cells divided about three times, whereas ~40% of pericentrin-depleted cells did not divide at all during the same time period (Fig. 3b). These two independent approaches demonstrate that G1 arrest can be induced from within G1, and that perturbation of mitotic events, such as spindle function and cytokinesis, are not required to trigger the arrest.

G1 arrest occurs concomitant with reduced Cdk2–cyclinA activity and requires p21

To more accurately determine the cell-cycle stage and molecular mechanism of the G1 arrest, we examined the levels and activity of Cdk–cyclin

complexes. Immunoprecipitations from pericentrin-depleted cells showed that Cdk2–cyclin A activity, but not cyclin A levels, was diminished in pericentrin-depleted cells compared with controls (Fig. 3c, data not shown). Similar results were obtained for several centrosome proteins (Fig. 3c). The diminished Cdk2 activity in the absence of a change in cyclin A levels suggested the presence of a Cdk inhibitory activity. In fact, human cells null for the Cdk inhibitor p21 suppressed the cell-cycle arrest (HCT116 *p21*^{−/−}, Fig. 3d), revealing a role for p21 in the inhibition of Cdk2–cyclinA complexes[19] and a molecular mechanism for the arrest.

G1-arrested cells have defects in centrosome structure and/or organization

Because disruption of centrosome proteins induced G1 arrest from within G1, we examined G1-arrested cells for defects in centrosome structure and organization. Immunofluorescence microscopy imaging using markers for centrioles and PCM revealed three categories of structural defects: centriole loss, centriole separation and what seemed to be centriole fragments. Importantly, all centrosome protein depletions that showed cell-cycle arrest, also showed centrosome defects in one or more of these categories when compared to controls (10 out of 10).
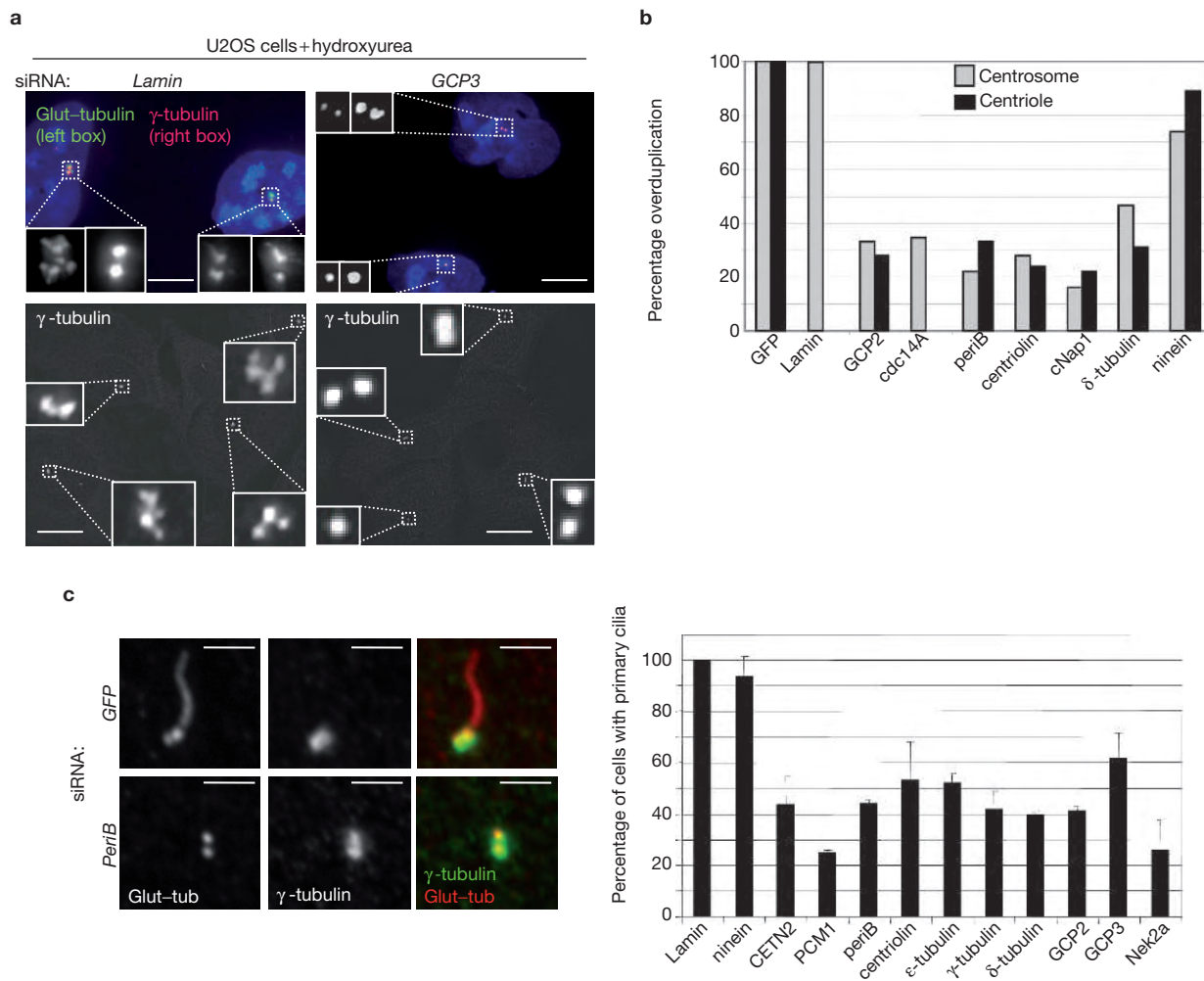
Figure 5 G1 arrested cells exhibit defects in centrosome function. (**a**) Immunofluorescence microscopy images of centrosomes in control (*lamin* siRNA) and *GCP3*-depleted U2OS cells after treatment with hydroxyurea (48 h) to induce S-phase arrest and supernumerary centrosomes. (**b**) The histogram shows numbers of centrosomes and centrioles in hydroxyurea-treated U2OS cells after siRNA treatment (64 h) against the indicated proteins. Overduplication: >2 γ-tubulin dots (centrosome); >4 centrin dots (centrioles). *n* = 200 cells per bar. Results are representative of three experiments. (**c**) Immunofluorescence microscopy images and quantification of primary cilia assembly in cells depleted of the indicated proteins. Cells were stained with an antibody to polyglutamylated tubulin (Glut-tub) and γ-tubulin. >200 cells per bar, normalized to 100% in control (lamin), an average of three experiments ± s.e.m. are shown. The scale bar represents 5 μm in **a** and 2 μm in **c**.

In Fig. 4A, we present data for the centrosome defect that exhibited the highest increase over control levels for each centrosome protein analysed (quantitative data for each of the three individual assays is shown in the Supplementary Information, Fig. S3). Some protein depletions induced defects in all three categories (for example, GCP2) and others in only one or two (see Supplementary Information, Fig. S3). For example, eight of nine centrosome protein depletions showed centriole loss (excluding ninein, see Supplementary Information, Fig. S3a) and six of nine showed separated centrioles (see Supplementary Information, Fig. S3b). Aberrant centrosomes did not result from cell-cycle arrest, as they were not detected in cells arrested by other means (for example, serum deprivation or hydroxyurea treatment).

The centriole-like fragments stained positively for centriole proteins (centrin) and often for a marker for stabilized tubulin (polyglutamylated tubulin), but not for PCM proteins (pericentrin and γ-tubulin, Fig. 4B). They were heterogeneous in size, usually smaller than centrioles and found in the vicinity of an intact centrosome. They were observed in four out of six centrosome protein depletions examined (see Supplementary

Information, Fig. S3c). A few of the same structures were examined at higher resolution by correlative electron microscopy[20]. This revealed elements that seemed to be disorganized microtubule-like structures (Fig. 4C). Two cells had three GFP–centrin dots and all seemed to be incomplete microtubule-like structures by electron microscopy. A third cell showed no structural correlates and could represent less complete centriole structures as previously described[20]. These data suggested that centrosome-protein depletion induced formation of centriole intermediates, in addition to the original centrioles that were analogous to abnormal centrioles produced by mutation of genes involved in centriole duplication[21,22]. Taken together, the results from these three structural assays show that centrosome defects accompany G1 arrest for all centrosome depletions analysed here, suggesting a strong link between these phenotypes.

G1-arrested cells exhibit defects in centrosome function

We reasoned that defects in centrosome structure and/or organization could perturb centrosome functions known to occur in G1, namely centrosome duplication and primary cilia assembly. In fact, previous studies

showed that loss of centrioles, as described above, was associated with improper centrosome duplication[23–25]. To examine defects in centrosome duplication, U2OS cells were treated with hydroxyurea to induce S-phase arrest and multiple rounds of centrosome duplication[26]. All centrosome protein depletions analysed that induced cell-cycle arrest and centrosome defects, also inhibited formation of supernumerary centrosomes and supernumerary centrioles (six out of six, Fig. 5a, b). In addition, supernumerary centrosomes and centrioles that occur naturally in U2OS cells (without added hydroxyurea) were reduced to more normal or lower than normal numbers (see Supplementary Information, Fig. S3d). These two assays show that centrosomes with abnormal structure were unable to duplicate properly.

We next examined the ability of centrosomes depleted of centrosome proteins to assemble primary cilia. Primary cilia are solitary microtubule-based structures that require functional centrosomes for their assembly[27], serve as environmental sensors and are implicated in human disease[28]. siRNAs targeting ten proteins that induced G1 arrest inhibited primary cilia formation (Fig. 5c). This loss of functional integrity was consistent with the observed defects in centrosome structure (Fig. 4A).

All centrosome protein depletions that lead to G1 arrest also showed defects in centrosome structure and organization, and centrosome function. This correlation is remarkable and suggests that defects in centrosome structure and function are tightly linked to centrosome-associated G1 arrest.

G1 arrest requires p53 and p38

We next investigated regulatory molecules and pathways that could control cell-cycle progression in centrosome protein depleted cells. Immunofluorescence microscopy imaging and biochemical strategies, demonstrated that the p53 tumour suppressor accumulated in nuclei of siRNA treated cells before G1 arrest (Fig. 6a) and that p53 did not translocate to nuclei in *lamin* siRNA-treated control cells (Fig. 6a). Translocation of p53 into the nucleus is consistent with its activation[29] and indicates a role for the protein in G1 arrest. Cell lines with compromised p53 (HCT116 *p53*^{-/-}, HeLa, Saos-2; Fig. 6b and see Supplementary Information, Fig. S4a) did not undergo G1 arrest, whereas cells with wild-type p53 arrested (RPE-1, BJ-1, HME-1 and HCT-116, Figs 1, 3d).

It is possible that p53-deficient cell lines acquire additional genetic changes that contribute to cell-cycle arrest in a p53-independent manner. To overcome this potential problem, we depleted *p53* acutely using siRNAs in cells concurrently depleted of centrosome proteins and found that G1 arrest was also suppressed under these conditions (Fig. 6c and see Supplementary Information, Fig. S4b,c). p53 activation is sometimes linked to DNA damage[30], but no evidence was found for DNA damage in cells depleted of centrosome proteins using an early marker for double-strand DNA breaks, 53BP1 (ref. 31). Robust 53BP1 staining was observed when DNA was damaged by etoposide or hydroxyurea (see Supplementary Information, Fig. S4d). These results demonstrate that G1 arrest induced by centrosome protein depletion is p53-dependent and occurs without detectable DNA damage.

p53 activity is modulated by multiple signal transduction pathways[32], including p38. p38 is a member of a pathway that responds to cellular stress and is linked to the cell cycle through senescence and differentiation pathways[33]. In cells depleted of centrosome proteins, p53 was
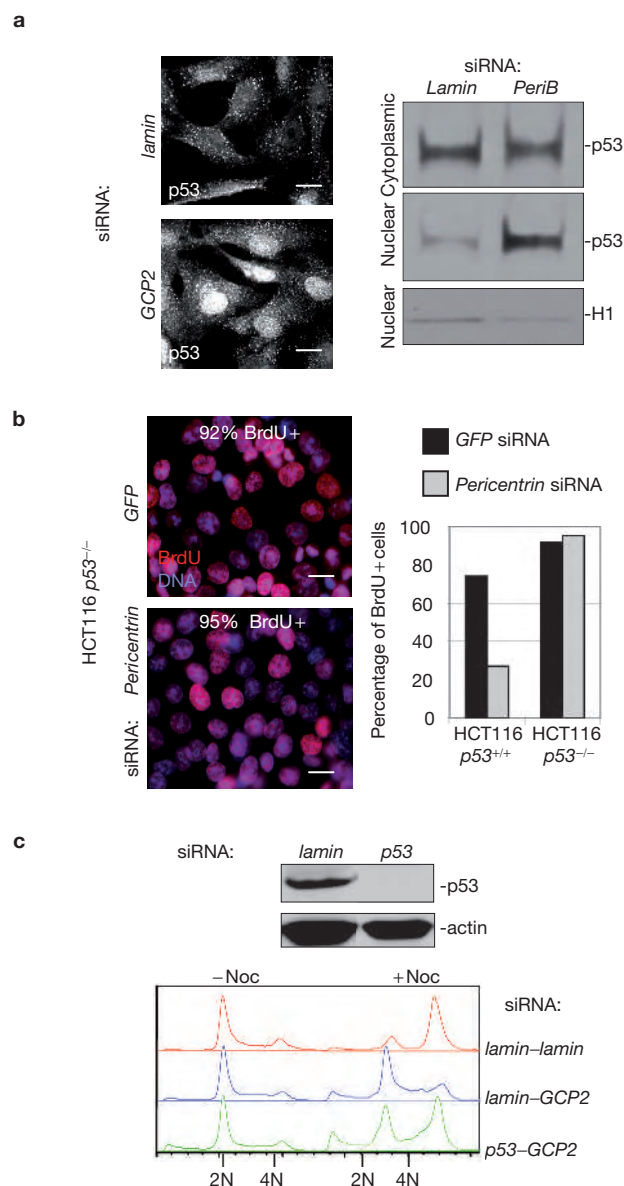
Figure 6 G1 arrest induced by centrosome protein depletion is p53-dependent. (**a**) Immunofluorescence microscopy images showing nuclear accumulation of p53 in *GCP2* siRNA-treated cells, but not controls. Immunoblots of cytoplasmic and nuclear extracts of siRNA-treated cells showing p53 nuclear accumulation after *pericentrin* siRNA treatment, but not after *lamin* siRNA treatment. Histone H1, loading control. (**b**) Immunofluorescence microscopy images and quantification of BrdU incorporation (16 h pulse) in *pericentrin* siRNA-treated cultures (60 h) of HCT116 *p53*^{-/-} cells. (**c**) Immunoblot from *p53* siRNA-treated and control cells. Actin, control. Flow cytometry profile of asynchronous (– noc) and nocodazole-treated (+ noc, 12 h) cells treated simultaneously with two siRNAs as indicated, >5000 cells per trace. Results are representative of at least two experiments. The scale bars represent 10 μm in **a** and **b**.

activated on Ser 33 (p53[P-Ser 33]), a residue known to be phosphorylated by p38 (Fig. 7)[34]. This phosphorylation was not observed in *ninein*-depleted or control siRNA treated cells (Fig. 7a). Consistent with the lack of detectable DNA damage in centrosome protein-depleted cells, we did not detect phosphorylation on Ser 15 of p53 by the DNA damage-associated ATM kinase (data not shown).
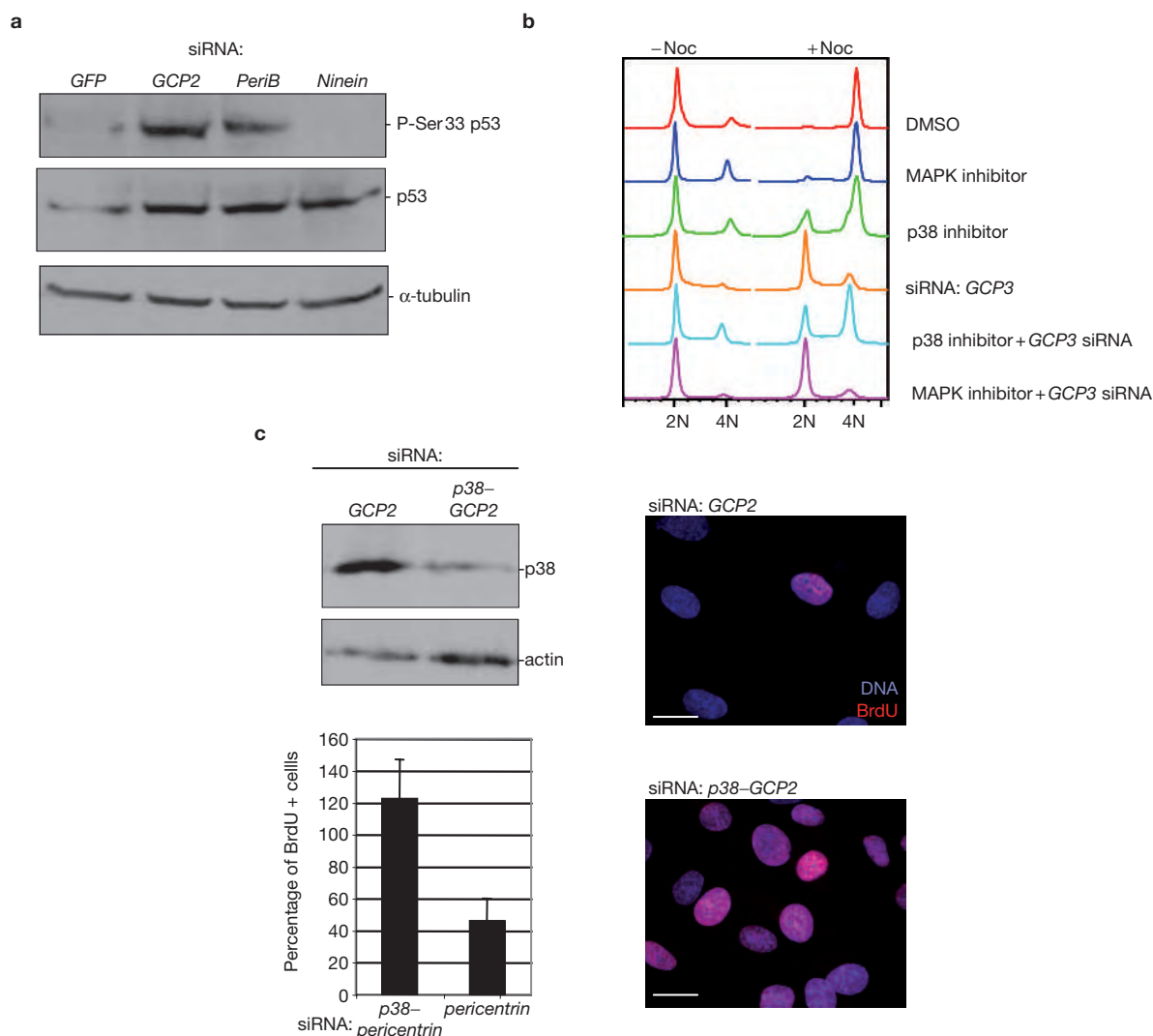
Figure 7 G1 arrest induced by centrosome protein depletion is p38-dependent. (a) Immunoblot showing an increase of phospho-Ser 33 p53 in cells treated with *GCP2* and *pericentrin* siRNAs. p53 and α-tubulin were used as loading control. (b) Flow cytometry profiles of inhibitor-treated cells and inhibitor-treated cells depleted of *GCP3* collected in the absence or presence of nocodazole (12 h). Results are representative of three experiments, >5000 cells per trace. (c) Western blot showing depletion of p38. The histogram shows quantification of BrdU positive cells (normalized to untreated) after depletion of pericentrin alone, or in tandem with p38. Results are representative of three experiments and are an average of three experiments ± s.d. Immunofluorescence microscopy images show BrdU incorporation in cells depleted of *p38* and *GCP2* or *GCP2* alone. The scale bar represents 10 μm.

We next examined whether p38 was required for the centrosome associated G1 arrest. We first showed that p38 was activated in PCM1-depleted cells by its upstream kinases (MKK3/6) through phosphorylation on Thr 180 and Tyr 182 (data not shown). We next showed that SB202190 and another inhibitor of the α and β isoforms of p38 (SB203580)[35] suppressed the G1 arrest (Fig. 7b) and reduced nuclear translocation of p53[P-Ser 33] if added before centrosome protein depletion, but not after (data not shown). In contrast, an inhibitor of the MEK signal transduction pathway (MAPK) had no effect on cell cycling. We confirmed pharmacological suppression of the G1 arrest by siRNA-mediated depletion of *p38α* (Fig. 7c).

The p38-activated form of p53 accumulates at centrosomes before G1 arrest

Immunofluorescence microscopy imaging demonstrated that before G1 arrest, the p38-phosphorylated form of p53 (p53[P-Ser 33])

concentrated at centrosomes in response to centrosome protein depletion but not control protein depletion (Fig. 8a). Centrosome accumulation of p53[P-Ser 33] occurred before its nuclear translocation (Fig. 8a), suggesting a multistep pathway for activation and nuclear entry of p53. Activated p38 was detectable at centrosomes in mitotic and most interphase cells (Fig. 8b). In summary, we show that p38 localizes to centrosomes, that p53[P-Ser 33] accumulates at centrosomes specifically in response to centrosome protein depletion and that both proteins are required for the centrosome-associated cell-cycle arrest. These observations are consistent with a role for p53 and p38 in transmitting signals from the centrosome to the cell-cycle machinery (p21–cyclinA–Cdk2 complexes) as part of a cell-cycle checkpoint that monitors changes in centrosome integrity and controls G1 to S phase progression. Consistent with this model are previous studies showing that p38 activates p53 (ref. 34), that p53 activates p21 (refs 36, 37) and that p21 inhibits Cdk2–cyclin complexes[19].

DISCUSSION

This study represents an extensive analysis of centrosome genes in vertebrate cells. We unexpectedly found that nearly all centrosome genes tested induced G1 arrest when depleted or overexpressed. Also unexpected was the observation that defects in centrosome structure and G1-centrosome functions always accompanied cell-cycle arrest, suggesting a link between these phenotypes. This work uncovers two functions common to many centrosome proteins (centrosome duplication and primary cilia assembly). We propose that these G1 functions are core functions of centrosomes in vertebrate cells. This study suggests that disruption of centrosome structure and/or function activates a 'centrosome damage' checkpoint that leads to G1 arrest.

Work from other studies supports the idea that cell-cycle progression is linked to centrosomes. Cell-cycle arrest was observed in cultured cells following centrosome disruption by laser ablation[6] or microsurgery[7], and in mouse oocytes following microinjection of centrosome antibodies[16]. Disruption of centrosome structure in all systems could logically lead to defects in centrosome functions such as duplication and primary cilia assembly, as described here. Links between premature centriole separation and aberrant centrosome duplication, and between defective centrosome duplication and cilia assembly, have been demonstrated previously[38]. Moreover, a recent paper also showed that siRNA depletion of *PCM1* and *pericentrin* induced p53-dependent cell-cycle exit[39]. Taken together, all these studies provide a strong link between defective centrosomes and cell-cycle arrest.

This study provides the first evidence for concordance between three distinct phenotypes — defective centrosome structure, centrosome dysfunction and G1 arrest. These observations provide strong support for a causal relationship between these phenotypes. We propose that cell-cycle arrest is triggered by defects in centrosome structure and/or function. Consistent with this idea, was the observation that the centrosome-bound fraction of targeted proteins was always reduced following centrosome protein depletion or expression of dominant-negative constructs. None of three phenotypes was observed following depletion of control proteins or ninein. The ability to induce G1 arrest from within G1 is consistent with disruption of centrosome and/or centriole structure and centrosome functions in G1. Finally, specific recruitment of regulatory molecules to centrosomes (p53) in response to centrosome protein depletion is consistent with a role for centrosomes in the cell-cycle arrest pathway.

The structural defects in centrosomes observed following depletion of centrosome proteins could arise through production of centrosome duplication intermediates that fail to mature, defects in parent centrosomes and/or centrioles that occur before G1, or the inability to remodel centrosomes during centrosome duplication[40]. We propose that most, if not all, centrosome proteins are required, perhaps as parts of an assembly line, to complete the construction of a functional centrosome. Master regulators likely control the overall process (for example, Plk4; refs 23, 25).

Our results suggest the presence of a novel cell-cycle checkpoint that prevents cells from entering S phase when they acquire defects in centrosome structure and/or function. Consistent with a checkpoint is the observation that centrosome-protein-depleted cells arrest with 'centrosome damage' (structural and/or functional defects) and re-enter the cell cycle only after target protein levels are restored. This mechanism of cell-cycle arrest, involving p53 recruitment to the centrosome may also apply to cells in which centrosomes have been physically altered[6,7], as these cells re-organize a functional microtubule-organizing centre that
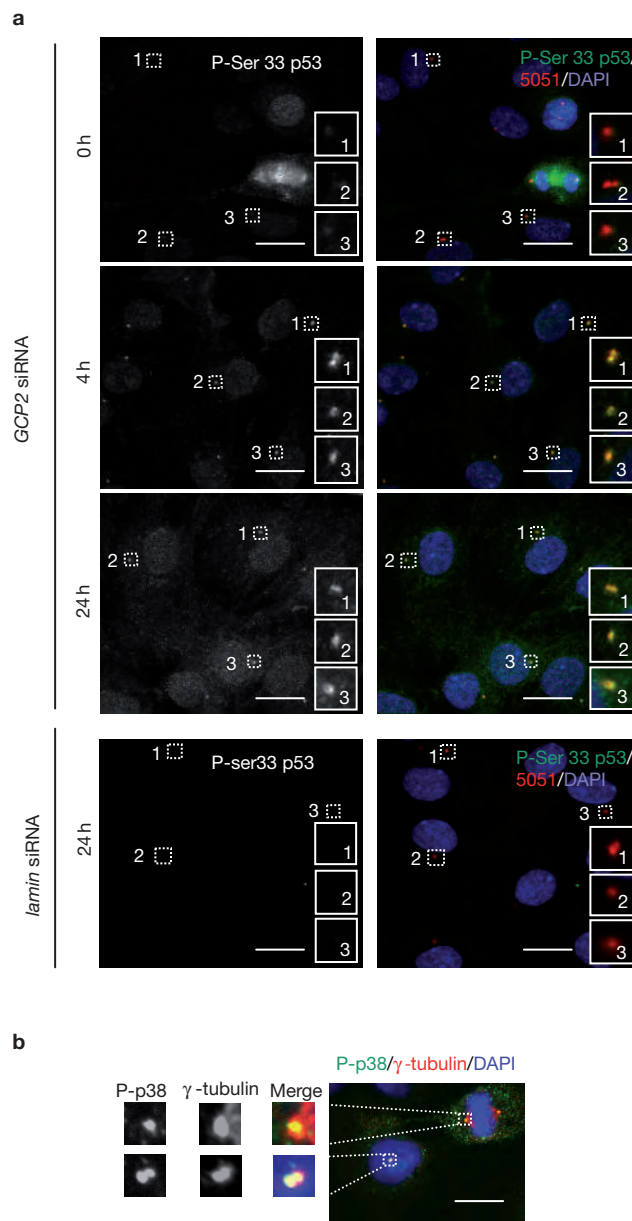
Figure 8 The p38-activated form of p53 accumulates at the centrosome before G1 arrest. (**a**) Immunofluorescence microscopy images showing p53[P-Ser 33] concentrated at centrosomes in response to centrosome protein depletion (*GCP2* siRNA) 48 h after siRNA but not control protein depletion (*lamin*). Kinetic experiments (times after release from serum starvation are indicated) show p53 concentration at centrosomes (4–24 h) before the nuclear staining becomes prominent (24 h). Magnifications of the numbered boxed areas are shown in the insets. 5051, centrosome marker. (**b**) Immunofluorescence microscopy images showing phospho-p38 at the centrosome of a mitotic (top inset) and interphase cell (bottom inset). The scale bar represents 10 μm in **a** and **b**.

lacks centrioles but contains pericentriolar material. As with other checkpoints, cell-cycle arrest can be overcome by depleting and/or inhibiting elements of the checkpoint pathway (for example, p53, p38 and p21). Our data thus satisfy the definition of checkpoint as having three elements: a change in a condition that is sensed by the cell (altered centrosome structure and/or function), a transducing system (p38 and/or p53) and a receiver element (p21).

Abrogation of the proposed centrosome-damage checkpoint has deleterious downstream consequences. In p53-deficient HeLa cells, depletion of proteins involved in centrosome duplication induces spindle defects, cytokinesis failure and aneuploidy[24,25]. In much the same way, p53-deficiency in many human tumours may abrogate the centrosome-damage checkpoint and contribute to centrosome defects, spindle dysfunction and aneuploidy[5,41].

DNA and centrosomes are semi-conservatively replicated once every cell cycle, their replication is initiated at the same cell-cycle stage (G1) and is controlled by some similar regulatory molecules[1]. In response to DNA damage, signalling molecules such as the DNA-damage checkpoint kinase, ataxia-telangiectasia mutated (ATM) are recruited to damaged DNA. We propose that in response to centrosome damage, activated p53[P-Ser 33] is recruited to centrosomes. *In vivo*, the centrosome-damage checkpoint could prevent cell cycling when centrosomes are compromised by pathogens[42–44] or other external perturbations (for example, heat; data not shown)[45]. Additional studies will be required to identify other centrosome-associated molecules of the checkpoint-control pathway, to determine how p53 and p38 are anchored at centrosomes and to uncover the precise mechanism of pathway activation. □

METHODS

Antibodies. We are indebted to the following investigators for providing antibodies: GCP2 and 3 (T. Stearns, Stanford, CA); ninein (G. Chan, Edmonton, Canada); Nek2a and cNap1 (A. Fry, Leicester, UK); pericentrin B (T. Davis, Seattle, WA); PCM-1 (A. Merdes, Edinburgh, UK); 20H5 Centrin-2 (J. Salisbury, Rochester, NY); cdc14A and cdc14B (P. Jackson, Stanford, CA); polyglutamylated tubulin (GT335) antibody (P. Denoulet, Paris, France); p53[P-Ser 33] (Y. Taya, Tokyo, Japan); and 53BP1 (T. Halazonetis, Geneva, Switzerland). Commercially available antibodies were also used: α-tubulin, γ-tubulin, ε-tubulin, actin, BrdU (Sigma, St Louis, MO); Ki-67 (BD Biosciences, Franklin Lakes, NJ); lamin A/C (Cell Signaling, Boston, MA); p38, histone H1, zyxin, Nek2, δ-tubulin, ε-tubulin (Santa Cruz Biotechnology, Santa Cruz, CA); phospho-p38 (Thr180/Tyr182, Cell Signaling); p53 (ab-2), p53 (ab-6). Pericentrin A/B[27] 5051 (ref. 46) and cyclins A2, B1, cdk1-2 (ref. 47) have been described previously.

Cell culture, siRNA, transfection and microinjection. These studies primarily used diploid, telomerase-immortalized RPE-1 cells (Clontech, Mountain View, CA)[48], cell lines containing wild-type p53 (BJ-1, HCT116, IMR-90, HCT116 *p21*^{-/-}) or compromised p53 (Saos-2, HeLa, HCT116 *p53*^{-/-}; HCT116 series, generous gift from B. Vogelstein, Baltimore, MD). HeLa and U2OS stably expressing GFP–centrin2 were prepared in our laboratory. Cells were grown as described by American Type Culture Collection (Manassas, VA). For G0–G1 synchrony, cells were grown for 24 h in media with reduced serum (0.25%) before experimentation. Targeted proteins were depleted with siRNAs delivered to cells at 1–200 nM using Oligofectamine or Lipofectamine 2000 (Invitrogen, Carlsbad, CA) per manufacturers instructions. Synthetic double-stranded siRNAs (Dharmacon, Lafayette, CO) were designed according to recently published suggestions[48] (for siRNA sequences see Supplementary Information, Fig. S1e). For rescue experiment siRNA, Smart pool siRNA localized in the UTR region was purchased from Dharmacon. Several genes were targeted with a second siRNA to eliminate possible non-specific effects. p38 (SB202190; Calbiochem, La Jolla, CA) and MAPK (PD98059) inhibitors were used at 10 μM and 50 μM respectively. For telophase microinjection experiments, cells synchronized in mitosis by 10 h nocodazole incubation (0.5 μg ml^{-1}), were microinjected into the nucleus 1 h after release, with either a plasmid encoding RFP–Peri[CT], or a control RFP plasmid using an Eppendorf transjector 5246 and Micromanipulator (Brinkman, Westbury, NY). Immediately after microinjection, cells were incubated with 10 μM BrdU for 24 h before fixation and staining. Alternatively, cells were transfected using calcium phosphate and incubated for 24 h with BrdU followed by fixation and staining.

Immunofluorescence microscopy and immunoblotting. Cells were prepared for immunofluorescence microscopy, imaged, deconvolved (Meta-Morph; Universal Imaging Corp., Downington, PA), displayed as two-dimensional projections of three-dimensional reconstructions to visualize the entire cell volume, and quantified as previously described[8]. Pixel intensity profiles (total intensity plot) were constructed from maximal intensity projections using Meta-Morph. Crude cell lysates were analysed for protein depletion. Cells were treated with siRNAs for 48–72 h, harvested and lysed in PBS supplemented with 1% Triton X-100 and a cocktail of protease inhibitors. Cell lysates were clarified at top speed in a microfuge for 15 min at 5 °C. Protein concentration for each lysate was determined using Bio-Rad protein dye reagent, loads were adjusted and proteins were resolved by SDS–PAGE and analysed by western blot. Uncropped images of key western blots are shown in the Supplementary Information, Fig. S5.

Kinase assays and nuclear fractions. Immunoprecipitations and kinase assays were performed as previously described[47]. Affinity-purified cyclin A antibodies were cross-linked to Sepharose beads and incubated with cell lysates (100–300 μg proteins) for 3 h before being washed four times in buffer. Precipitated proteins were resuspended in 20 μl 1× SDS–PAGE sample buffer for immunoblot analysis. For kinase assays, immunoprecipitation beads were dissolved in 5 μl kinase buffer with 10 mM DTT and 20–50 μM ATP. Each sample was incubated with 5–10 μCi γ-^{32}P-ATP (#BLU-502A; PE/NEN Life Sciences, Boston, MA) and 1.5 μg histone H1 (#1004875; Roche, Basel, Switerland) in a final volume of 16 μl for 30 min at 30 °C. Reactions were terminated with 8 μl 5× SDS–PAGE sample buffer and processed for autoradiography and quantified by phosphorimage analysis (Storm 820, Molecular Probes, Carlsbad, CA). Nuclear fractions were prepared by resuspending trypsinized cells in cold nuclear extraction buffer (320 mM sucrose, 5 mM MgCl₂, 10 mM HEPES, 1% Triton X-100 at pH 7.4) by gentle vortexing, followed by incubation on ice for 10 min. Nuclei were then pelleted at 2,000g and washed twice with nuclei extraction buffer without Triton X-100. Nuclei yield and integrity were confirmed by microscopic examination. All washes were combined to obtain the cytoplasmic fraction and both fractions were processed for immunoblot analysis (as above).

Flow cytometry, BrdU assay and proliferation assay. Cells treated with siRNAs for 48–72 h were exposed to 1–5 μg ml^{-1} nocodazole for 12 h, removed from plates and fixed in ethanol. Cells stained with propidium iodide were analysed by flow cytometry (FACSCAN, Becton Dickinson, Franklin Lakes, NY) using FlowJo software (Tree Star, Inc., Ashland, OR). BrdU labelling was performed essentially as previously described[49]. Cells were incubated with 10 μM BrdU for 16–24 h before fixation in 4% formaldehyde for 2 min and post-fixation in 100% methanol. Generational tracking of cell populations by flow cytometry was accomplished by labelling cellular proteins with 2 μM carboxyfluoroscein diacetate, succinimidyl ester (CFDA-SE, Molecular Probes) for 5 min according to the manufacturers' specifications, so that each cell division results in halving of the total cellular fluorescence.

Centrosome duplication, primary cilia formation and quantification of centrosome defects. After 24 h of siRNA-mediated depletion of centrosome proteins, U2OS cells were blocked in S-phase by incubation in hydroxyurea-containing growth media (4 mM) for an additional 40 h before fixation in 100% ice-cold methanol. To unambiguously identify centrioles in interphase, cells were pre-treated with nocodazole (5 μg ml^{-1}) for 2 h or incubated on ice for 30 min to depolymerize microtubules before fixation. Centrioles from untreated U2OS cells in interphase and mitosis were also counted. The indicated antibodies were used to detect centrosomes and centrioles. Primary cilia were detected as described using GT335 antibody[27]. Briefly, cells were retransfected 48 h after the first siRNA transfection using Oligofectamine. At 72 h, primary cilia were induced by culturing RPE1 cells in medium with 0.25% serum for 48 h. To quantify centrosome defects (Fig. 4A), three categories of centrosome defects were an analysed (structure, separation and loss). For each depleted centrosome protein, the assay showing the largest difference compared with control was plotted as the fold difference and compared with ninein.

Note: Supplementary Information is available on the Nature Cell Biology website.

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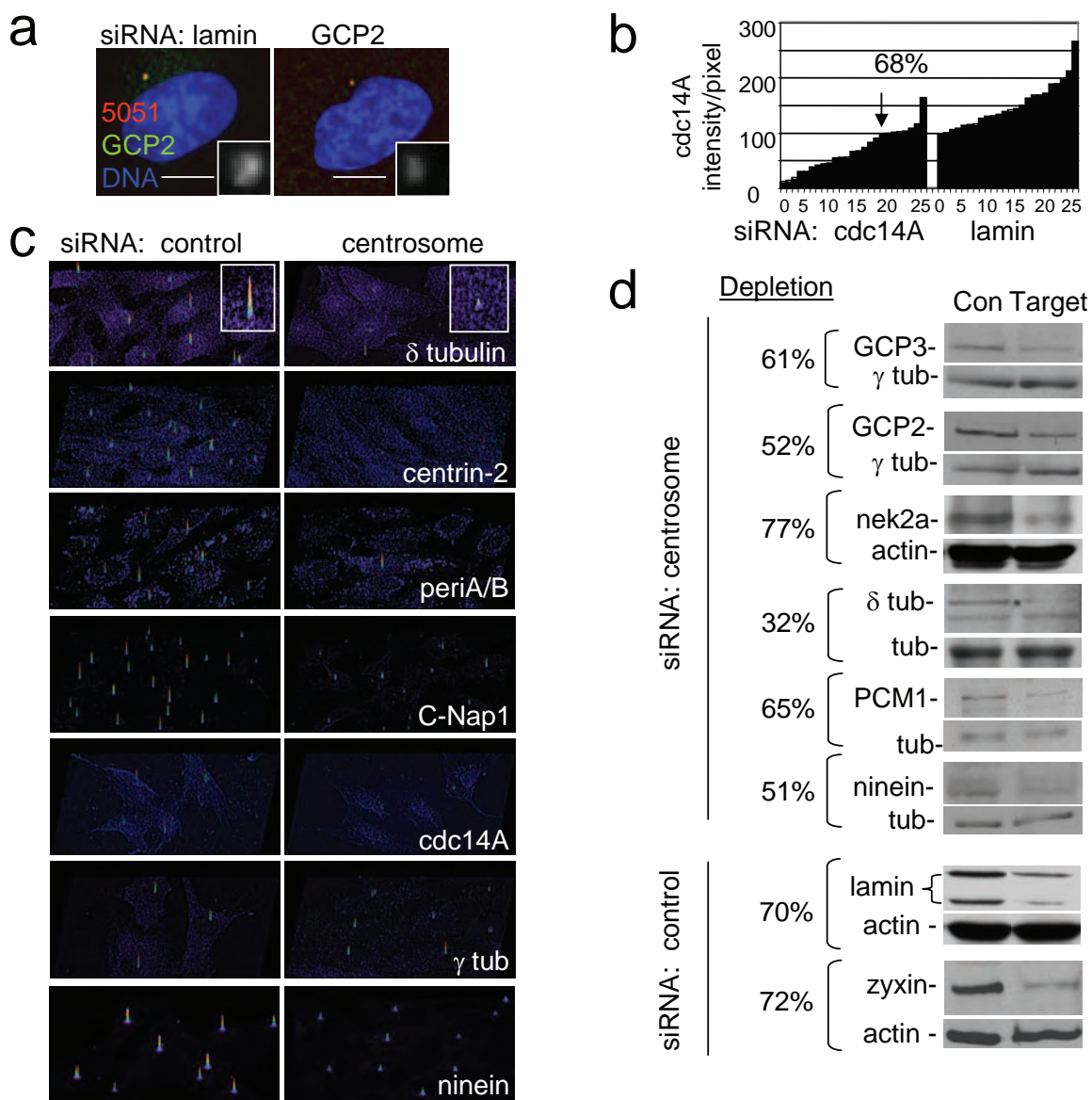
e

Control siRNA sequences used:

GFP	CAUGAAGCAGCACGACUUC
LAMIN A/C	CUGGACUUCCAGAAGAACA
KER18	CAUCAAGGUCAAGCUGGAC
ZYXIN	UGUGGCUGUCAACGAACUC

Centrosome protein siRNA sequences used:

CENTRIOLIN	GGAUCAGAGACUCUACCUU	
PERI A/B	GAAGGAGAAGGAGACGGCA	
PERI A/B.2	GCAGCUGAGCUGAAGGAGA	(Shown throughout paper)
PERI B	UUGGAACAGCUGCAGCAGA	(Shown throughout paper)
NINEIN	GUGCUGCAGCAGACAUUAC	
NINEIN.2	UAUGAGCAUUGAGGCAGAG	
GAMMA	UGACCGCAAGGACGUCUUU	
GCP2	UCUCGUACUCCAGAAGACU	
GCP2.2	AGAUCGAGAAGGCGUUUAA	
GCP3	UGCAGCAUCUGCUUCUCUU	
GCP5	ACUUCGCCUGGUCCAACUU	
CNAP1	UCUAUCCGAAAGCCCAGUC	
CNAP1.2	CUGUCACUCAAGCCAAGGA	
PCM1	GUCCCCCAAACAGAGAAAC	
PCM1.2	UCAGCUUCGUGAUUCUCAG	
DELTA TUB	GGUUCUGGAAACAACUGGG	
NEK2	AGGGAACCAAGGAAAGGCA	
EPSILON TUB	AGUCGGCAGAGCACUGUGA	
CDC14A	GCACAGUAAAUACCCACUA	
CDC14A.2	CCGUGCGUAUCUCGCUUAA	
CDC14B	GCAAAUGCUGCCUUCCUUG	
CENTRIN2	GAGCAAAAGCAGGAGAUCC	(Shown in Figure 2)
CENTRIN2	SmartPOOL UTR region	(Shown in Figure 2)

Other siRNA sequences used:

P53	GGACAAGGGUUGGGCUGGG
P53.2	AGACUCCAGUGGUAAUCUA
P38	UGUGAUUGGUCUGUUGGAC
P38.2	CCAAAUUCUCCGAGGUCUA

Figure S1 siRNAs specifically deplete centrosomal proteins. **a,** Immunofluorescence images show reduction of GCP2 protein at the centrosome 72h after GCP2 siRNA treatment compared to control (lamin). Inset : 5X magnification of centrosomal GCP2 signal. 5051, centrosome marker. Scale bar: 5μm **b,** Graph displays the average fluorescence intensity/pixel of cdc14A at individual centrosomes (bars) in siRNA-treated cells (72h). Arrow shows that 68% of cdc14A siRNA-treated cultures are below the lowest control levels. **c,** Semi-quantitative pixel intensity profiles, constructed from optically sectioned (Z-axis) fluorescence images of cells treated with siRNAs targeting lamin (left, control) or centrosomal proteins (right, as indicated), shows that siRNAs deplete targeted proteins at the centrosomes . **d,** Western blots from siRNA-treated cultures (72h) probed for the targeted protein (Target) or control (Con, usually lamin), as indicated. Actin, γ tubulin, or pan-specific tubulin immunoblots demonstrate equivalent loading conditions as indicated (lower panels of each set). **e,** siRNAs sequences used to target centrosome proteins. Underlined sequences are presented in the paper.

a

No G1 arrest

G1 arrest

siRNA (# of cells without nocodazole/# of cells with nocodazole)

mock (15k/15k events) keratin 18 (15k/15k)

γ tub (15k/15k) Gcp3 (12.4k/8.5k)

GFP (15k/9.6k) ninein (15k/15k)

periB (15k/14.4k) cdc14B (15k/15k)

lamin (15k/15k) Zyxin (15k/15k)

C-nap1(15k/11.6k) ε tub(15K/15k)

Gcp2 (6k/7k) PCM1 (9.5k/12k)
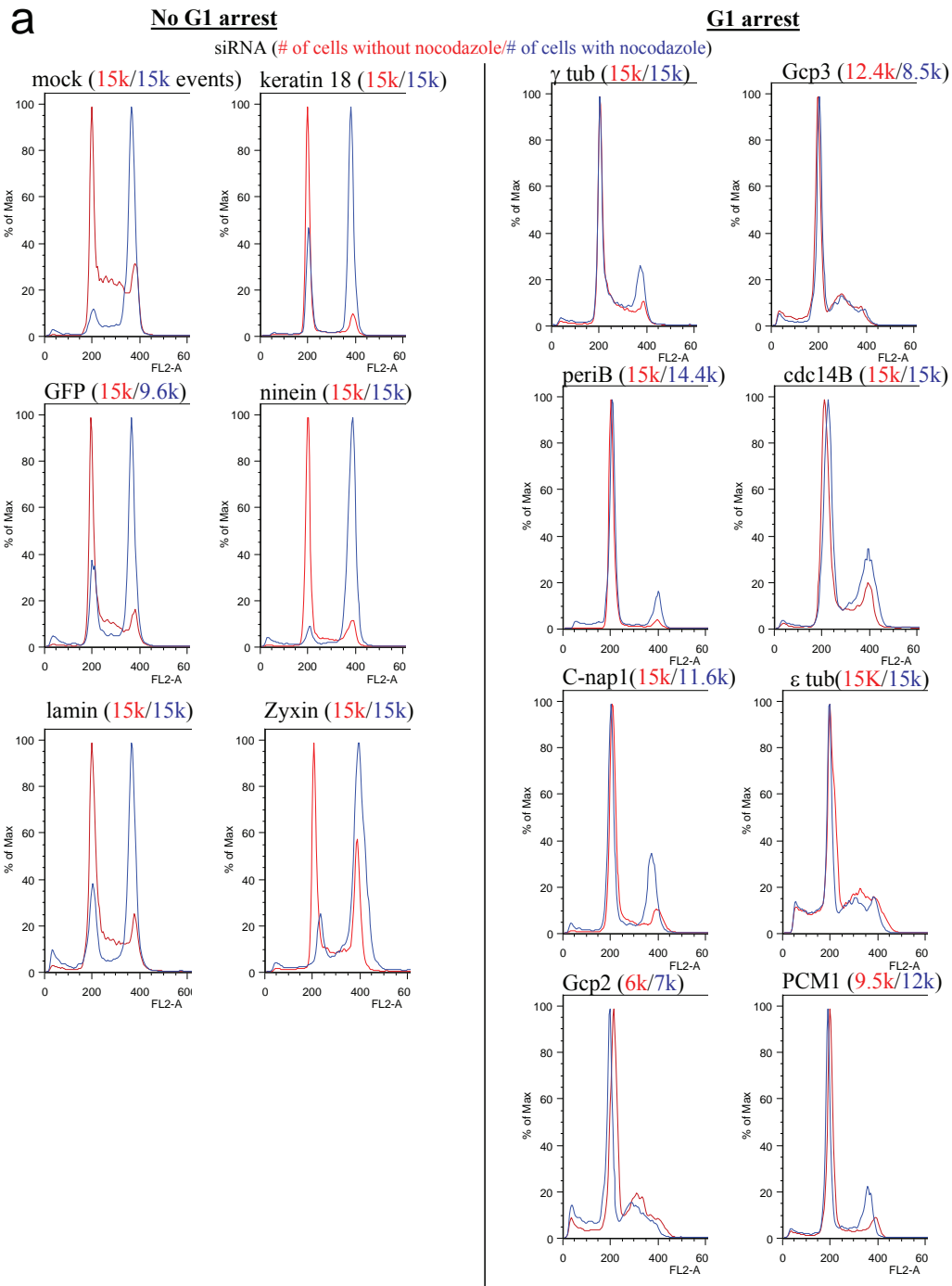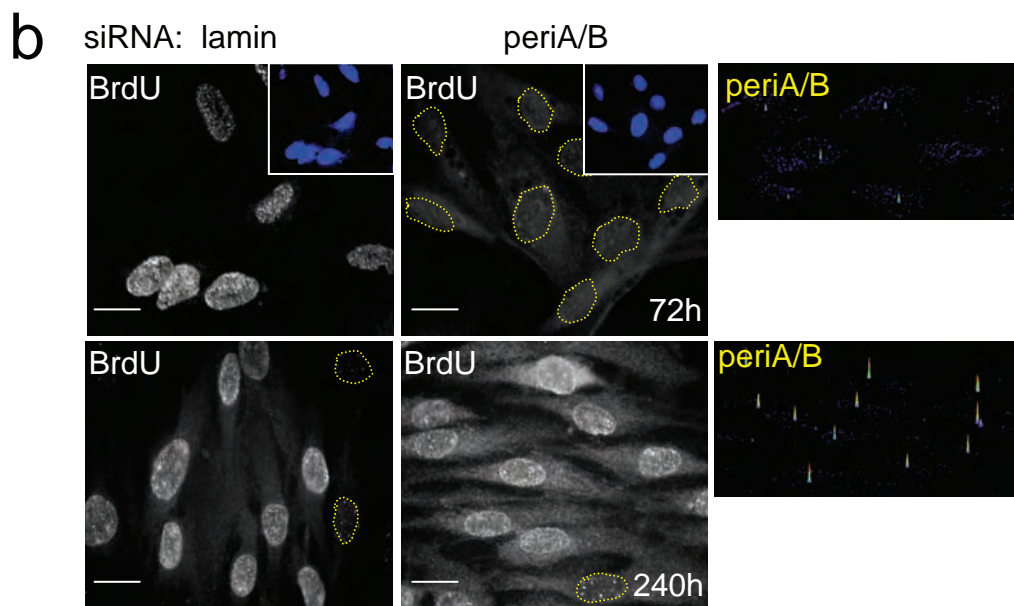
Figure S2 a, Cell cycle arrest is induced after centrosome protien depletion. Flow cytometry traces of RPE cells treated with indicated siRNAs. Red, no nocodazole; blue, + nocodazole. The number of cells examined are shown in parentheses where the colors indicate -noc/+noc. See Fig.1 for details. **b,** Cell cycle arrest induced by pericentrin depletion (periA/B, 72h, upper panel) is relieved upon return to normal protein levels (periA/B, 240h, lower panel). BrdU incorporation in cells treated with lamin or pericentrin-specific siRNAs for 72 or 240 hours. Centrosome pixel intensity profiles (right panels) show that centrosomal levels of pericentrin returns to normal (top) when cells begin cycling and incorporate BrdU. Scale bar: 10μm. Insets: DAPI stain. Dotted lines: nucleus.
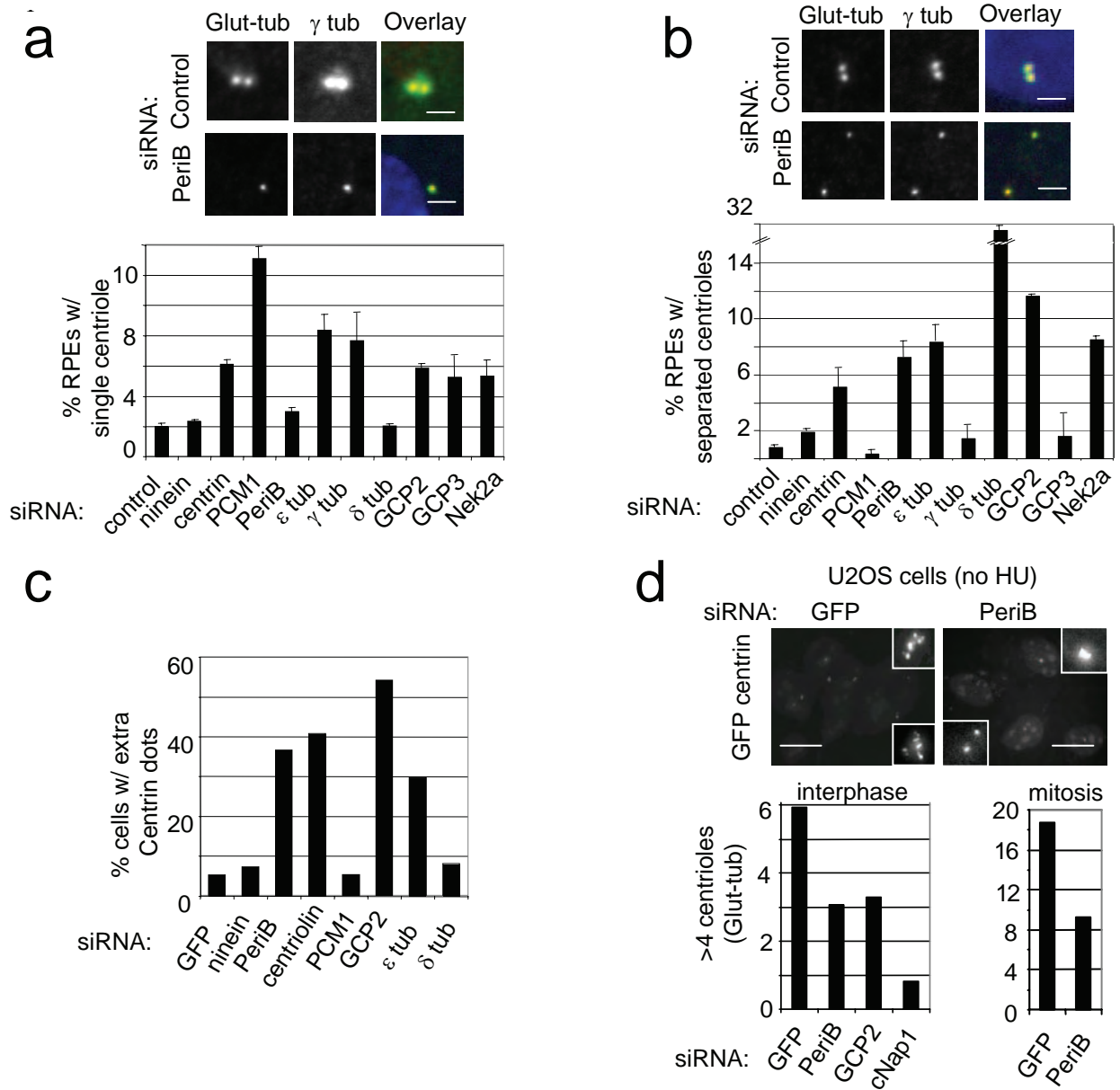
Figure S3 Structural and functionnal defects induced in RPE cells after centrosome protein depletion. **a,** Immunofluorescence images and quantification of RPE cells with single centriole (stained with polyglutamylated-tubulin, Glut-tub) after siRNA-depletion of the indicated proteins and serum withdrawal. Scale bar: 2µm. Average of 2 experiments ± SEM. >200 cells /bar. **b,** Immunofluorescence images and quantification of RPE cells with separated centrioles (stained with Glut-tub) after siRNA-depletion of the indicated proteins and serum withdrawal. Scale bar: 2µm. Average of 2 experiments ± SEM. >200 cells /bar. **c,** Quantification of GFP-centrin expressing RPE cells with extra GFP-centrin structures (presumed centriole intermediates) induced in RPE cells after siRNA treatment as indicated. >200 cells/bar. **d,** Defects in centrosome duplication leads to reduction of centriole number in U2OS cells in the absence of hydroxyurea (HU). Immunofluorescence images of centrosomes in control (GFP siRNA) and periB siRNA-depleted non-hydroxyurea-treated U2OS cells showing centriole number reduction (GFP-centrin). Quantification of centriole reduction (Glut-tub) in interphase (left) and mitotic (right) U2OS cells following siRNA treatment of the indicated proteins. Scale bar: 10µm. >100 interphase cells or >150 mitotic cells/ bar.

a

b

c

d
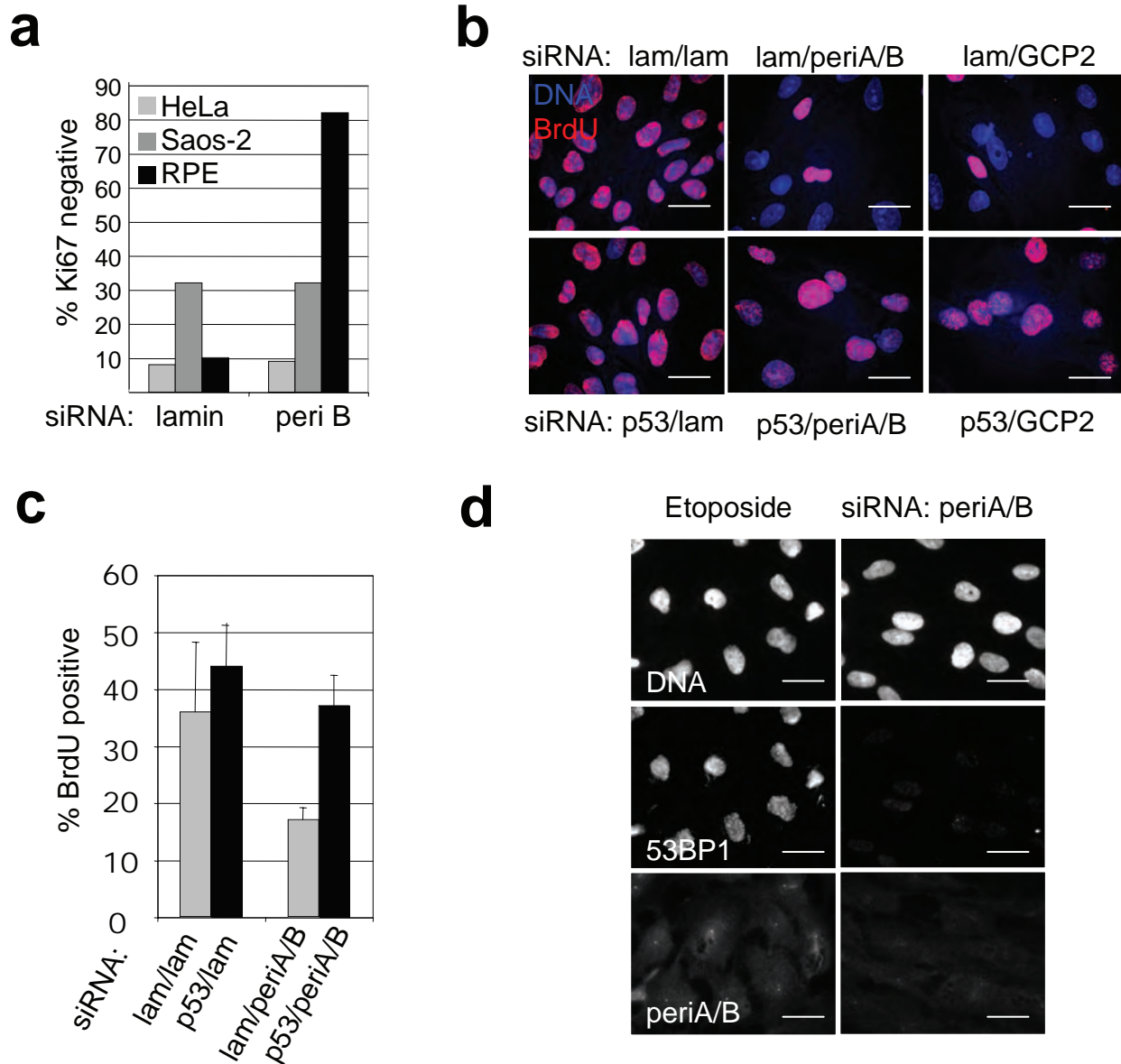
Figure S4 G1 arrest is p53-dependent and occurs without detectable DNA damage. **a,** p53-deficient cells (HeLa, Saos) do not arrest. Graph shows percentage of Hela, Saos-2, and RPE cells staining negatively for Ki67 following depletion of lamin or pericentrin B (peri B). n=1000 cells/bar **b,** p53 depletion suppresses cell cycle arrest in RPE cells. BrdU incorporation in cells pretreated with siRNAs directed against either lamin or p53 for 24h, then treated with indicated siRNA (after backslash) for 72 hour. BrdU was added 24h before processing**.** Scale bar: 10μm. **c,** Quantification of cell cycle arrest suppression after p53 depletion. BrdU incorporation in cells pretreated with siRNAs directed against either lamin or p53 for 24h, then treated with siRNA (after backslash: lam, periA/B). Average of 3 experiments +/- SD. **d,** Cell cycle arrest occurs without detectable DNA damage. p53BP1 is not affected following centrosome protein depletion. Cell cultures treated with either etoposide (positive control) or pericentrin depleted cells were stained with an antibody directed against p53BP1, to reveal damaged DNA. Co-staining of pericentrin (periA/B) included to show specificity of protein depletion. Scale bar: 10μm.

a.
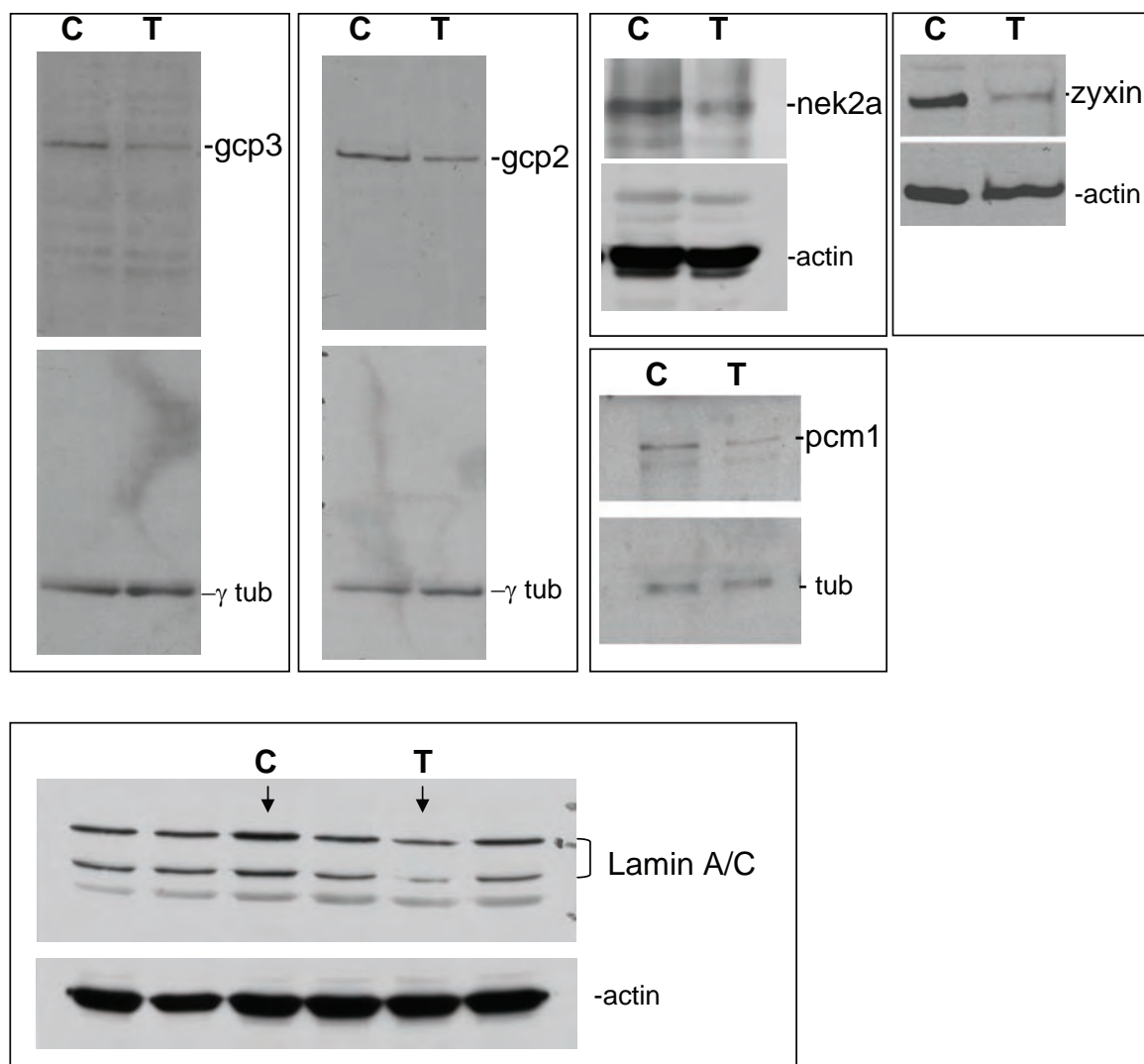
Figure S5 Western blots shown in full **a,** Western blots from Supplemental Fig1. C: control siRNA, T: target siRNA **b,** Western blots from Fig. 3c. **c,** Western blots from Fig. 6c . **d,** Western blots from Fig 7a. **e,** Western blots from Fig. 7c. C: control siRNA, T: target siRNA

Survivin Modulates Microtubule Dynamics and Nucleation throughout the Cell Cycle ⊡ ⊻

Jack Rosa,* Pedro Canovas,† Ashraful Islam,† Dario C. Altieri,†‡ and Stephen J. Doxsey*‡

*Program in Molecular Medicine and †Department of Cancer Biology and the Cancer Center, University of Massachusetts Medical School, Worcester, MA 01605

Survivin is a member of the chromosomal passenger complex implicated in kinetochore attachment, bipolar spindle formation, and cytokinesis. However, the mechanism by which survivin modulates these processes is unknown. Here, we show by time-lapse imaging of cells expressing either green fluorescent protein (GFP)-α-tubulin or the microtubule plus-end binding protein GFP-EB1 that depletion of survivin by small interfering RNAs (siRNAs) increased both the number of microtubules nucleated by centrosomes and the incidence of microtubule catastrophe, the transition from microtubule growth to shrinking. In contrast, survivin overexpression reduced centrosomal microtubule nucleation and suppressed both microtubule dynamics in mitotic spindles and bidirectional growth of microtubules in midbodies during cytokinesis. siRNA depletion or pharmacologic inhibition of another chromosomal passenger protein Aurora B, had no effect on microtubule dynamics or nucleation in interphase or mitotic cells even though mitosis was impaired. We propose a model in which survivin modulates several mitotic events, including spindle and interphase microtubule organization, the spindle assembly checkpoint and cytokinesis through its ability to modulate microtubule nucleation and dynamics. This pathway may affect the microtubule-dependent generation of aneuploidy and defects in cell polarity in cancer cells, where survivin is commonly up-regulated.

INTRODUCTION

Survivin is a member of the inhibitor of apoptosis (IAP) gene family (Salvesen and Duckett, 2002), which is overexpressed in nearly every human tumor and frequently associated with resistance to therapy, and unfavorable outcome (Altieri, 2003). Experimental work carried out in vitro (Beltrami et al., 2004) and in transgenic animals (Grossman et al., 2001; Okada et al., 2004) has assigned a dual function to survivin: protection from apoptosis and regulation of cell division. Although the cytoprotective function of survivin has recently come into better focus (Blanc-Brude et al., 2003; Marusawa et al., 2003) and has been linked to the upstream initiation of mitochondrial apoptosis (Dohi et al., 2004), the mechanism by which survivin participates in cell division is still unclear. Although survivin-like IAP molecules in model organisms seem to participate predominantly or exclusively in cytokinesis (Uren et al., 1999; Speliotes et al., 2000), reduction or loss of survivin in mammalian cells has been associated with a panoply of cell division defects that include supernumerary centrosomes (Li et al., 1999), aberrant spindle assembly (Giodini et al., 2002), mislocalization of mitotic

kinases (Wheatley et al., 2001), loss of mitotic checkpoint(s) (Lens et al., 2003), and cytokinesis failure with appearance of multinucleated cells (Li et al., 1999). Adding further complexity to its potential role in mitosis, survivin localizes to multiple sites on the mitotic apparatus, including centrosomes, microtubules of the metaphase and central spindle, kinetochores, and midbodies (Fortugno et al., 2002).

Previous experiments of antibody microinjection suggested a potential role of survivin in spindle microtubule assembly, reflected in a phenotype of flattened mitotic spindles depleted of microtubules (Giodini et al., 2002). Similar observations were reported in knockout studies, and homozygous deletion of survivin resulted in nearly complete absence of mitotic spindles (Okada et al., 2004), and appearance of disorganized tubulin bundles (Uren et al., 2000). This model may fit well with the observation that survivin forms a complex with some of the chromosomal passenger proteins, notably, Aurora B (Adams et al., 2001) and the more recently described Borealin/hDasra B (Gassmann et al., 2004; Sampath et al., 2004). It has been proposed that the chromosomal passenger complex is a regulator of kinetochore attachment and cytokinesis (Adams et al., 2001) and is important for bipolar spindle formation in a pathway independent of Ran-GTP involving Aurora B-dependent phosphorylation of the microtubule-destabilizing Kin I kinesin MCAK (Gass-mann et al., 2004; Sampath et al., 2004). Survivin can enhance the activity of Aurora B (Bolton et al., 2002), suggesting a model whereby survivin regulates spindle formation through Aurora B.

In this study, we examined the role of survivin in microtubule dynamics and its potential dependence on the chromosomal passenger complex. Using time-lapse live imaging of two independent microtubule markers, the microtubule plus-end protein EB1 and α-tubulin, we found that survivin

functions as a novel regulator of microtubule dynamics and nucleation in interphase and throughout mitosis and that this pathway is independent of Aurora B activity.

MATERIALS AND METHODS

Cells and Cell Cultures

Cervical carcinoma HeLa cells and monkey COS-7 cells were obtained from the American Type Culture Collection (Manassas, VA) and were maintained in culture according to the manufacturer's specifications. Diploid, telomerase-immortalized human RPE-1 cells (hTERT-RPE-1) were obtained from Clontech (Palo Alto, CA) (Morales *et al.*, 1999).

Antibodies

Antibodies used were α-tubulin (#DM1a; Sigma-Aldrich, St. Louis, MO), acetylated-tubulin (#6-11B-1; Sigma-Aldrich), β-galactosidase (#1083 104; Roche Diagnostics, Indianapolis, IN), tyrosinated-tubulin (rabbit W2) (Gurland and Gundersen, 1995), survivin (Fortugno *et al.*, 2002), anti-EB1(catalog no. 610534; BD Biosciences, Franklin Lakes, NJ), anti-γ-tubulin (HM2569, polyclonal peptide raised against amino acids AATR; Covance, Princeton, NJ) and hemagglutinin (HA) (#3F10; Roche Diagnostics). As secondary antibodies, we used anti-mouse cy3 (Molecular Probes, Eugene, OR), anti-rabbit cy5 or fluorescein isothiocyanate (FITC) (Jackson ImmunoResearch Laboratories, West Grove, PA), or anti-rat cy3 (Molecular Probes).

Microinjection and Live Cell Imaging

COS-7 cells were synchronized by double-thymidine block (Quintyne and Schroer, 2002), and upon release from the S phase block, they were microinjected into the nucleus with plasmids containing HA-survivin or β-galactosidase (200 ng/ml) together with 25 ng/ml an EB1-GFP plasmid (a gift from L. Cassimeris, Lehigh University, Bethlehem, PA) using an Eppendorf transjector 5246 and Micromanipulator (Brinkmann, Westbury, NY). Cells were returned to the incubator for 8–10 h and then used for live imaging or fixed and stained for other antigens as indicated using methods described previously (Gromley *et al.*, 2003).

Adenoviral Transduction

The replication-deficient adenoviruses encoding survivin (pAd-survivin) or GFP (pAd-GFP) were described previously (Mesri *et al.*, 2001). Cells (2.5 × 10^7) were transduced at a multiplicity of infection of 50 for 24 h at 37°C, washed, and replenished with fresh growth medium for further analysis.

Microtubule Quantification in Fixed Cells

COS-7 cells plated at comparable density were transduced with pAd-GFP or pAd-survivin, treated with 10 μM of the microtubule-depolymerizing agent nocodazole (Sigma-Aldrich), and harvested at increasing time intervals between 5 and 60 min (Hergovich *et al.*, 2003). Cells were fixed in ice-cold methanol, washed three times in phosphate-buffered saline with 1% bovine serum albumin and 0.5% Triton X-100 (PBSAT), and stained with antibody to acetylated tubulin (Gromley *et al.*, 2003) followed by a secondary anti-IgG conjugated to cy3 and mounting on glass slides (Prolong Antifade; Molecular Probes). Microtubule fluorescence was quantified by acquiring 13 optical sections (333 ± 50 nm) using wide-field fluorescence microscopy (Olympus, Tokyo, Japan) and a 63× objective from six random fields. For individual cell measurements, we obtained fluorescence values of areas outside those containing microtubules and subtracted this background from the total value (typically 5–10% of total cellular fluorescence). The fluorescence intensity (integrated optical density) (Dictenberg *et al.*, 1998) for every optical section in every full cell profile or within an entire population was calculated using MetaMorph software (Universal Imaging, Downingtown, PA) (see above) or IP Lab software version 3.5.4 (Scanalytics, Fairfax, VA) as described previously (Purohit *et al.*, 1999; Gromley *et al.*, 2003). These values were then averaged to calculate the fluorescence of the total population.

RNA Interference (RNAi)

Double-stranded (ds)RNA oligonucleotides targeting survivin (S4), Aurora B, or a control unrelated sequence (VIII) were described previously (Altieri, 2003; Beltrami *et al.*, 2004). Cells were transfected with 50 nM of the various dsRNA oligonucleotides using Oligofectamine (3 ml/well) reagent (Invitrogen, Carlsbad, CA) and replenished with growth medium after 4 h. After 24–48 h, in the absence of any detectable apoptosis, cells were harvested and analyzed for reduction of survivin levels by immunoblotting, or used for live analysis of EB1-GFP or GFP–α-tubulin dynamics.

Microtubule Regrow Assay (Nucleation)

Cells grown on coverslips were either transfected with the indicated dsRNA oligonucleotides or transduced with the indicated adenovirus as described above. Cells were than incubated for 4 h in 8 μM nocodazole at 37°C and then for an additional 30 min on ice before washing. Coverslips were than washed twice in 50 ml of ice-cold phosphate-buffered saline and incubated at 37°C for 2 min to allow microtubule growth and subsequently fixed in −20°C methanol, washed three times in PBSAT, and stained with antibodies to EB1 and γ-tubulin followed by cy3 and FITC-conjugated secondaries, respectively. EB1 foci were quantified by acquiring 16 optical sections (200 ± 50 nm) using wide-field fluorescence microscopy (Olympus) and a 100× objective from random fields. EB1 foci were individually counted from the entire z-series, and final images are presented as maximum projection of all planes used. Centrosomes containing two γ-tubulin foci were used. Graphs represent data taken from 25 to 30 cells in two experiments.

Live Microscopy of GFP-EB1

Cells (COS-7 or RPE) were plated on coverslips (25 mm in diameter) and were placed in a chamber (PDMI-2; Harvard Apparatus, Holliston, MA) in complete medium with CO$_2$ exchange (0.5 l/min) at 37°C. Cells were imaged every 3 s for two or more minutes using a 100× objective on an inverted microscope (Olympus IX-70). Images were captured on a CoolSNAP HQ charge-coupled device camera (Roper Scientific, Trenton, NJ). Time-lapse movies of EB1 movements were obtained. In some cases, individual images were concatenated to produce linear elements representing the total distance traveled and providing a measure of the total amount of microtubule growth during the imaging period (MetaMorph software 4.6). Using similar visualization techniques, we quantified the number of growing microtubules in cells. Where indicated, 10 μM taxol (Sigma-Aldrich) was added to live microscopy media. Resulting movies are shown at a rate of 15 frames per second.

Time-Lapse Analysis of Cells Expressing GFP-α-Tubulin

RPE cells stably expressing GFP-α-tubulin were transfected with survivin-specific S4 or control VIII dsRNA oligonucleotides and imaged by confocal laser scanning microscopy. Microtubule growth, catastrophe, shrinking, and rescue as well as growth/catastrophe transition rates for individual microtubules were calculated from images collected as time-lapse movies from several random areas of the cytoplasm that in most cases comprised the leading edge of the cell. Average transition values were obtained from five microtubules. Similar results were obtained from five individual cells in two different experiments. For targeting of Aurora B kinase, RPE GFP-α tubulin cells were first transfected with control (VIII) or Aurora B-directed dsRNA oligonucleotide for 36 h and analyzed by Western blotting. In independent experiments, cells were treated with the Aurora B kinase inhibitor hesperadin (100 nM for 6 h) characterized in previous studies (Hauf *et al.*, 2003; Sessa *et al.*, 2005). For analysis of microtubule dynamics, cells prepared as described above were observed using an inverted Zeiss microscope equipped with a 100×, numerical aperture 1.4 objective lens, a spinning-disk confocal scan head (PerkinElmer Life and Analytical Sciences, Boston, MA), and a MicroMAX interline transfer cooled charge-coupled device camera (Roper Scientific). All images (16-bit) were acquired using a single-wavelength (488-nm) filter cube. Image acquisition was controlled by Ultraview RS software (PerkinElmer Life and Analytical Sciences). Time-lapse sequences were acquired at 3-s intervals by using an exposure time of 0.2 s at four optical planes per interval with a Z-step of 0.3 μm. Resulting movies are shown at a rate of 15 frames per second.

Quantification of Microtubule Dynamics

Individual microtubules were analyzed as described previously (Rusan *et al.*, 2001). Briefly, time-lapse images were exported from the proprietary Ultraview software and imported into MetaMorph software (Universal Imaging) for further analysis. A stack of four optical planes was used to make a z-projection at each time point, and a time-lapse movie was reconstructed. The position of the microtubule end was tracked using the "track points" function in MetaMorph that was linked to Excel to produce a history plot of each microtubule. Growth and shortening phases were identified based on the history plots. The frequency of catastrophe was calculated by dividing the sum of the number of transitions from growth to shortening and pause to shortening by the sum of the duration of growth and pause. The frequency of rescue was calculated by dividing the sum of the number of transitions from shortening to growth and shortening to pause by the duration of shortening. Microtubule dynamicity was calculated as the total number of tubulin dimers exchanged at the microtubule end (using 1624 dimers/μm), considering the lifetime of the microtubule (Waterman-Storer *et al.*, 2000; Toso *et al.*, 1993). The value from each microtubule was used to calculate an average for each experiment and was used in Table 1. The time spent in each phase (shrink, growth, and pause) was recorded, and the percentage of time spent in each phase was calculated for each microtubule. The percentage of time was averaged individually and used in Table 1.

Immunofluorescence of Phosphorylated Histone H3

RPE-GFP-α-tubulin cells were grown on glass coverslips, treated with control or 100 nM hesperadin for 6 h, and fixed in −20°C methanol for 30 min. Coverslips were stained with 4,6-diamidino-2-phenylindole (DAPI) and an antibody to phosphorylated H3 (catalog no. 6-570; Upstate Biotechnology, Lake Placid NY) followed by cy5 secondary reagents. Images were acquired using the MetaMorph software as described above.

Quantification of Phosphorylated H3 Fluorescence

The method used was similar to the quantification EB1-GFP fluorescence (supplemental material). Briefly, 0.2 μm optical sections were taken of each mitotic cell for DAPI, GFP, and cy5. Because phosphorylated H3 specifically labels the chromatin, a region of the cytoplasm was used as background to subtract from the cy5 fluorescence. The DAPI labeling for each corresponding phosphorylated H3 image was used to define a region of interest based on the "threshold image" function in MetaMorph. The region was transferred to the appropriate phosphorylated H3 image, and the fluorescence in the defined region (occupied by the chromatin) was quantified.

Statistical Analysis

Data were analyzed using the unpaired t test on a GraphPad software package for Windows (Prism; GraphPad Software, San Diego, CA). Phosphate-buffered saline values of 0.05 were considered statistically significant.

RESULTS

Depletion of Survivin by RNA Interference Increases Microtubule Dynamics

Because of its reported localization to microtubules (Fortugno *et al.*, 2002) and its ability to alter microtubule organization during mitosis (Giodini *et al.*, 2002), we asked whether survivin modulated microtubule dynamics in living cells. We first examined microtubule dynamics in cells depleted of survivin by small interfering RNAs (siRNA). Transfection of RPE cells with a previously characterized dsRNA oligonucleotide targeting survivin (S4) (Beltrami *et al.*, 2004) resulted in >90% reduction in survivin levels by immunoblotting 48 h later, whereas a control dsRNA oligonucleotide (VIII) had no effect on survivin levels (Figure 1A). We next examined several parameters of microtubule dynamics, including microtubule growth, catastrophe (transitions from growth to shrinking), shrinking, rescue (transitions from shrinking to growth) and pause (periods between growth and shrinking) in interphase cells stably expressing GFP-labeled α-tubulin (Desai and Mitchison, 1997). We found that the average frequency of catastrophe (number per second) and dynamicity (dimers exchanged at the tip/lifetime of microtubule; see *Materials and Methods*) (Waterman-Storer *et al.*, 2000; Toso *et al.*, 1993) were significantly higher in survivin-depleted cells (Figure 1, C and E) compared with control cells (Figure 1, B and D; n > 25 microtubules from 5 cells, 2 experiments/condition); other parameters were not significantly different from controls. Each parameter of microtubule dynamics is independently presented in Table 1. Histories of growth and shrinking events of five microtubules per condition are shown in Figure 1, B–E, and in Movies 1 and 2. In addition, we analyzed cultures by fluorescence microscopy with an antibody to acetylated tubulin, a posttranslationally modified tubulin found in stabilized microtubules (Bulinski *et al.*, 1988). The acetylated tubulin signal was diminished compared with controls (our unpublished data; see below). The decrease in acetylated tubulin staining and increased frequency of microtubule catastrophe demonstrate that survivin depletion increases microtubule dynamics.

To independently validate results obtained with GFP-α tubulin-expressing cells, we used GFP-tagged EB1 as a marker for the plus ends of growing microtubules. Recent studies have shown that GFP-EB1 accurately reflects microtubule growth rates and the number of growing microtubules, including those nucleated from centrosomes (Piehl *et al.*, 2004; Tirnauer *et al.*, 2004). Stable expression of GFP-EB1 in control cells (VIII) revealed GFP-EB1 foci moving outward from the centrosome as previously described (Movie 3) (Piehl *et al.*, 2004). The number of GFP-EB1 foci in survivin-depleted cells was increased compared with control cells (Figure 2, A–C). When EB1 foci were collectively dis-
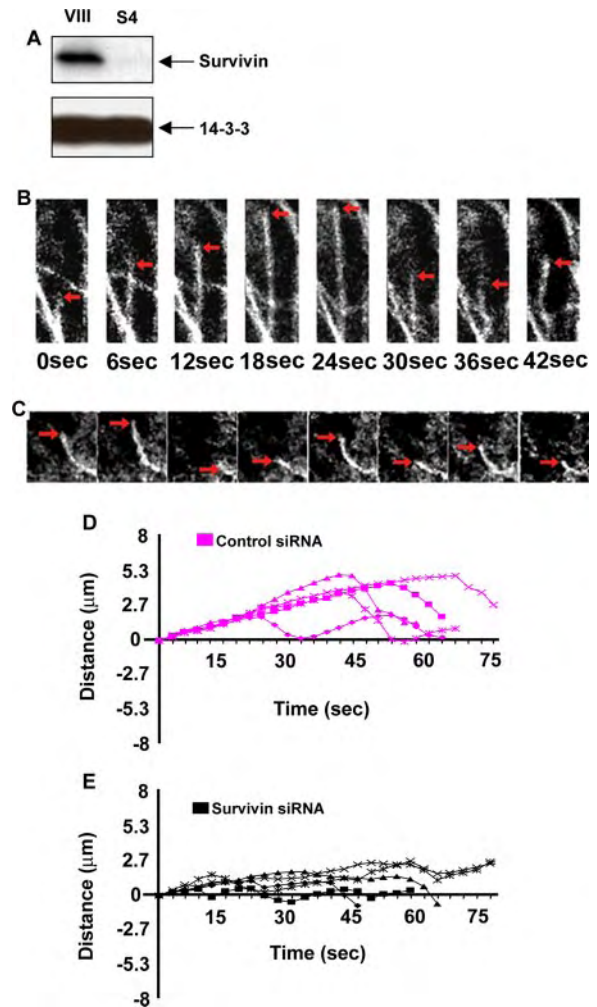
Figure 1. Survivin silencing increases microtubule dynamics. (A) RPE cells were transfected with survivin-specific S4 or control VIII dsRNA oligonucleotides (siRNAs), harvested after 48 h, and analyzed by immunoblotting. (B and C) Analysis of microtubule growth and shrinking. RPE cells stably expressing GFP-α tubulin were transfected with control VIII (B) or survivin-specific S4 dsRNA oligonucleotide (C), and imaged by time-lapse videomicroscopy (see Movies 1 and 2). The extent of growth and shrinking (red arrows) was measured for individual microtubules in interphase. (D and E) Quantification of microtubule dynamics observed in B and C. The length of microtubule polymer growth or shrinking (distance in micrometers) was examined over time in control cells (D) or survivin-depleted cells (E). Increasing values represent microtubule growth; decreasing values represent shrinking. Transitions from growth to shrinking (catastrophe), shrinking to growth (rescue), periods of no net growth (pause), and other parameters of microtubule dynamics are quantified in Table 1. Symbols represent five individual microtubules from two cells for both D and E.

played as a single projected image in control cells, long tracks representing extended periods of microtubule growth were observed (Figure 2, D, F, and H; Movie 3). Survivin-depleted cells expressing similar levels of GFP-EB1 (see below) had shorter EB1 tracks (Figure 2, E, G, and H; Movie 4). These results are consistent with an increase in the number of growing microtubules and a higher rate of catastrophe (Gliksman *et al.*, 1993). The changes in microtubule parameters observed in survivin-depleted cells occurred in the absence of changes in total cellular α-, β-tubulin levels (our

Table 1. Microtubule dynamics in survivin-depleted cells

A

	Avg. frequency rescue (s^{-1})	Avg. frequency catastrophe (s^{-1})
Control siRNA	0.070 ± 0.0148	0.025 ± 0.0047
Survivin siRNA	0.094 ± 0.0207	0.045 ± 0.0044*
Aurora B siRNA	0.064 ± 0.0464	0.023 ± 0.0103
Hesperadin analogue	0.064 ± 0.0270	0.030 ± 0.0082
Hesperadin	0.069 ± 0.0168	0.027 ± 0.0120

B

	Avg. time (%)	Avg. growth rate (μm/s)	Avg. shrink rate (μm/s)	Dynamicity (dimer/s)
Control siRNA				
Shrink	24.67	0.383 ± 0.1167	0.920 ± 0.2752	686.38
Pause	20.42			
Growth	54.56			
Survivin siRNA				
Shrink	27.94	0.398 ± 0.1258	0.852 ± 0.2503	811.89*
Pause	19.80			
Growth	52.43			
Aurora B siRNA				
Shrink	27.94	0.326 ± 0.0598	0.817 ± 0.2480	587.11
Pause	19.85			
Growth	52.21			
Hesperadin analogue				
Shrink	30.38	0.338 ± 0.1215	0.897 ± 0.1715	634.55
Pause	21.94			
Growth	47.68			
Hesperadin				
Shrink	22.77	0.391 ± 0.0592	0.897 ± 0.4081	618.12
Pause	19.80			
Growth	57.42			

(A) Catastrophe is increased in survivin-depleted cells compared with cells treated with control siRNAs or siRNAs targeting Aurora B (1.66- to 1.99-fold increase, respectively; value indicated by asterisk). (B) The average time microtubules spent shrinking, pausing, or growing is not significantly perturbed. There is no significant difference in growth or shrinkage rates in survivin-depleted cells compared with controls. Dynamicity is increased in cells with depleted survivin. Here, dynamicity represents the exchange of dimer at the microtubule tip over time (seconds); see *Materials and Methods*. Pauses represent periods between growth and shrinking or vice versa (<0.5-μm change). All data shown were acquired from 25 microtubules in five cells per experimental condition. All differences described are statistically significant (*t* test; p < 0.005).

unpublished data). These results confirm data from GFP-α-tubulin-expressing cells and verify that GFP-EB1 is a reliable marker for microtubule dynamics.

The increase in the number of EB1 foci demonstrated that more microtubules were present in survivin-depleted cells and suggested an increase in the number of microtubules nucleated from centrosomes. To test this directly, cells were treated with nocodazole to depolymerize microtubules and then washed to remove the drug and to allow regrowth of centrosomal microtubules. By counting the number of EB1 foci stained with anti-EB1 emanating from centrosomes, an accurate determination of microtubule nucleation could be determined as described previously (Piehl *et al.*, 2004; Tirnauer *et al.*, 2004). We found a significant increase in the number of EB1 foci after siRNA-mediated depletion of survivin compared with cells treated with a control siRNA (Figure 3A), demonstrating an increase in the number of centrosome-nucleated microtubules.

Expression of Survivin Suppresses Microtubule Dynamics and Nucleation at Multiple Cell Cycle Phases

Based on the increase in microtubule dynamics and nucleation observed in survivin-depleted cells, we reasoned that elevated survivin levels would suppress these parameters. To test this prediction, we first examined GFP-EB1 movements in living COS-7 cells microinjected with a plasmid encoding GFP-EB1 together with a plasmid encoding HA-survivin or a control protein, β-galactosidase. As expected, multiple GFP-EB1 foci emanated from the centrosome in control β-galactosidase-expressing cells (Figure 4, A and B, and Movie 5) and long GFP-EB1 tracks marking EB1 movements over time were observed (Figure 4, B and D). When the same cell was subsequently treated with taxol to suppress microtubule dynamics, GFP-EB1 movements were abolished, and no GFP-EB1 foci or tracks of EB1 movements were detected (Figure 4C and Movie 6). Survivin-expressing cells revealed a phenotype similar to that of taxol-treated cells. In many cells (~80%), no detectable GFP-EB1 foci were observed and GFP-EB1 track projections yielded little to no linear dimension (Figure 4E and Movie 7). GFP-EB1 levels achieved in these experiments were roughly similar in all cells examined (±11%; see below) and never approached levels known to induce microtubule changes (Ligon *et al.*, 2003). Consistent with the decrease in the number of GFP-EB1 foci, the number of centrosomal EB1 foci and hence the
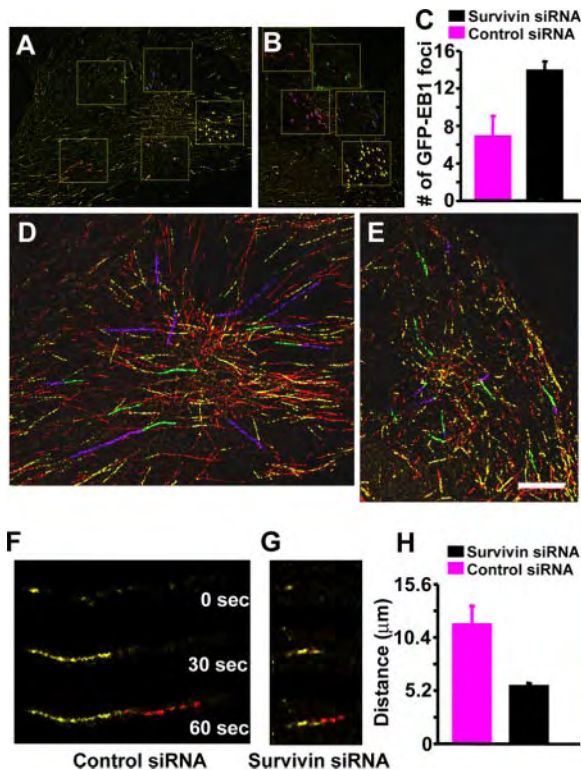
Figure 2. Survivin silencing increases the number of growing microtubules and decreases the duration of microtubule growth. (A and B) Number of EB1 foci. GFP-EB1-expressing RPE cells were transfected with control dsRNA (A) or survivin-specific S4 dsRNA (B), and EB1 foci were examined in composite images made from three consecutive frames of each movie (see Movies 3 and 4). (C) GFP-EB1 foci, representing individual microtubules, are quantified from fields covering 50–70% of a cell's area (boxes in A and B) from five cells in two separate experiments (C; each bar represents the average number of EB1 tracks). (D and E) GFP-EB1 movements. GFP-EB1-expressing RPE cells were transfected with control dsRNA (D) or survivin-specific S4 dsRNA (E), and EB1 movements were examined over 1-min (see Movies 3 and 4). All GFP-EB1 movements, representing microtubule growth, are displayed as linear tracings in D and E. The first 15 s of microtubule growth is represented in yellow, and the final 45 s is in red. Bar (E), 5 μm for D and E. Examples of microtubules used for analysis are displayed as green to blue instead of yellow to red. (F and G) Higher magnification images of individual growing microtubules in D (control; VIII transfectants) and E (survivin; S4 transfectants) at times indicated. (H) Quantification of GFP-EB1 tracking distances after transfection of dsRNA VIII (control) or survivin-directed S4 (survivin) oligonucleotide. Length in micrometers. Data represent 10 measurements from each of 10 cells from two separate experiments. Examples of microtubules analyzed for H are shown as green (first 15 s) and blue (next 45 s) in D and E. Bars (C and H) represent the mean ± SD.

number of microtubules nucleated from centrosomes was decreased (Figure 3B).

We next examined the effect of elevated survivin levels in mitotic cells, where microtubules are more dynamic than interphase cells (Rusan *et al.*, 2001). We used living COS-7 cells overexpressing either survivin or β-galactosidase (control), and expressing similar levels of GFP-EB1. We found that survivin-expressing cells had significantly less spindle-associated GFP-EB1 and more cytoplasmic GFP-EB1, which was reversed in control cells (Supplemental Figure 1). Moreover, survivin-expressing cells showed little to no detectable microtubule growth, because most spindles (85%; n = 20)
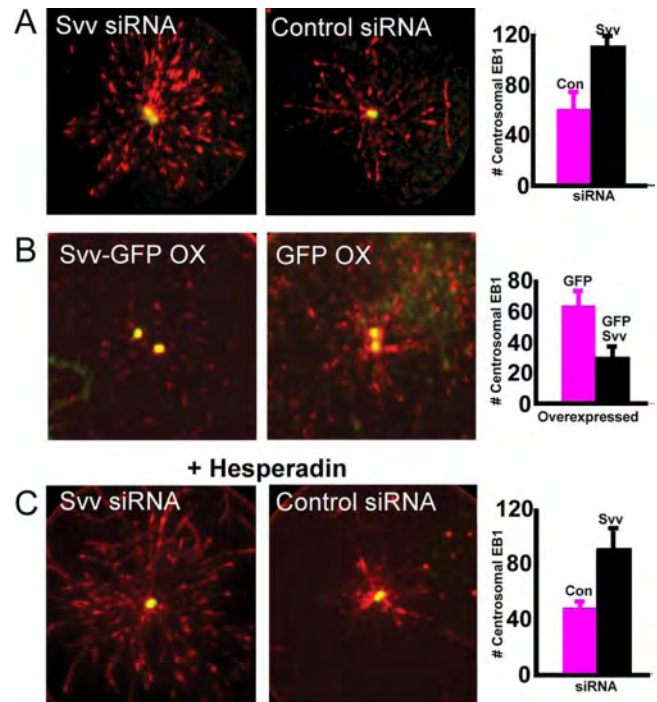
Figure 3. Survivin modulates the number of growing microtubules emanating from the centrosome. (A) Maximum projection of z-series taken of individual centrosomes in interphase RPE cells treated with nocodazole and then allowed to regrow microtubules for 2 min. Cells had been treated with either survivin-specific S4 dsRNA (A; left) or control dsRNA (A; right). Cells were stained by immunofluorescence using an antibody to EB1 (red) and γ-tubulin (green) (A; left and right), and foci were quantified in each z-plane and compared with adjacent planes to ensure that individual foci were not counted multiple times (graph; A, far right). (B) Maximum projection as in A where cells had been transduced either by pAd-GFP-survivin (B; left) or pAd-GFP-survivin (B; right). Cells were stained by immunofluorescence and quantified as in A (B; far right). (C) Maximum projection as in A and B where cells had been treated with either survivin-specific S4 dsRNA (C; left) or control dsRNA (C; right) in the presence of hesperadin. Cells were stained by immunofluorescence and quantified as in A and B (C; far right).

contained a negligible number of organized GFP-EB1 foci (Figure 4, F and G, and Movie 11). Although EB1 foci were present in some spindles (~15%; n = 20), their number never exceeded 10% of control levels (our unpublished data). As expected, control cells showed multiple GFP-EB1 foci moving away from both spindle poles (Figure 4, F and H, and Movie 10). Results from living cells were also confirmed in fixed cells. In control experiments, fixed β-galactosidase-expressing cells contained organized bipolar spindles with numerous EB1 foci (Figure 4, I–K). Conversely, fixed cells expressing survivin had little to no spindle-associated GFP-EB1 foci (Figure 4, L–N) and revealed small or disorganized mitotic spindles as reported previously (Giodini *et al.*, 2002).

We next examined the effect of survivin on microtubule dynamics during cytokinesis. In control cells expressing β-galactosidase (Figure 5, A–C), GFP-EB1 foci in midbodies were numerous (Figure 5A). They moved away from the center of the midbody (the zone that does not stain for microtubules at asterisk; Figure 5D, arrow; Gromley *et al.*, 2005) as well as toward the midbody center (Figure 5E, arrow), showing that microtubules were growing in both
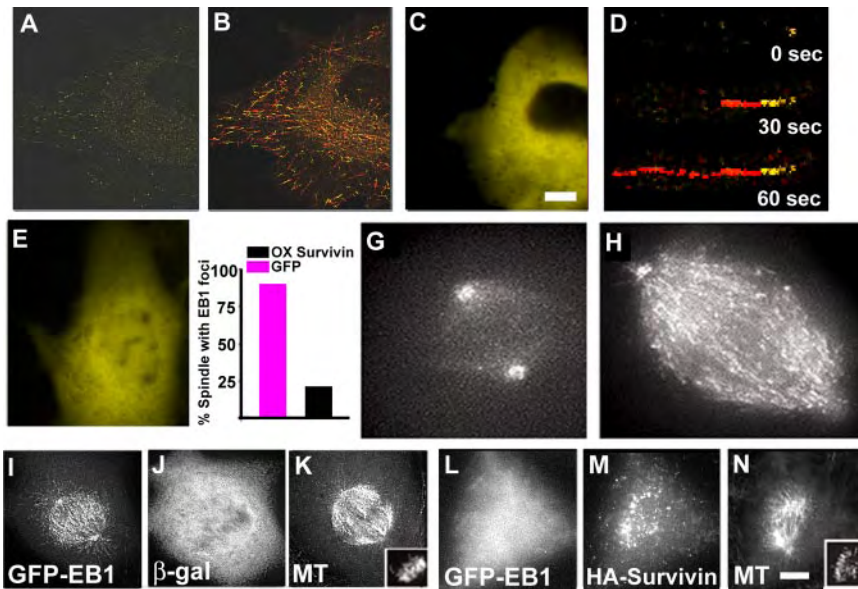
Figure 4. Increased levels of survivin suppress microtubule growth in interphase and mitotic cells. (A and B) Individual frames from a movie of GFP-EB1 in an interphase COS-7 cell coexpressing β-galactosidase (control) showing multiple foci at time 0 (A) that became more visible after growth (displayed as tracks that extend for long distances over 1 min; B; see Movie 5). First 15 s of microtubule growth are in yellow, and last 45 s are in red. (C) Taxol treatment. The same cell as in B treated with 10 μM taxol for 31 min; no detectable GFP-EB1 foci are seen (see Movie 6). (D) Higher magnification of a growing microtubule in B at 0, 30, and 60 s of filming. (E) Suppression of microtubule dynamics by survivin. Cell coexpressing survivin and GFP-EB1 showing no detectable GFP-EB1 foci (see Movie 7). (F) Quantification of spindles with detectable GFP-EB1 foci (or short tracks) in living cells expressing β-galactosidase or survivin. Data are the average of two separate experiments. n > 20 cells per bar. (G and H) Individual frames from movies of GFP-EB1 in mitotic cells expressing either survivin (G; see Movie 11) or β galactosidase (H; see Movie 10). Bar (C), 5 μm for A–H. (I–N) Cells coexpressing GFP-EB1 together with β-galactosidase (I–K) or survivin (L–N) were analyzed for GFP-EB1 foci (I and L), β-galactosidase and HA-survivin expression (J and M), or microtubules (K and N). Insets, DNA labeled with DAPI. For all injection studies, an average of 91% of cells survived injections, and 96% of those expressed both GFP-EB1 and either survivin or β-galactosidase at levels that do not affect microtubule dynamics or organization (survivin). Bar (N), 10 μm for I–N.

directions at this site (better visualized in Movies 8 and 9). These data suggest that midbody microtubules are highly dynamic, of dual polarity, and undergo bidirectional growth both toward and away from the midbody center. In comparison, GFP-EB1 foci in survivin-expressing cells (Figure 5, F and H) were either undetectable or diminished in number. The expressed survivin (Figure 5G) primarily accumulated with the endogenous protein at the midbody (Li *et al.*, 1999), suggesting that this was the site of action of the ectopically expressed protein. Consistent with previous observations (Uren *et al.*, 1999; Speliotes *et al.*, 2000), survivin-expressing cells with reduced microtubule dynamics often failed cytokinesis and generated multinucleated cells (our unpublished data).

Expression of Survivin Stabilizes Microtubules in Interphase and Mitosis

Because of its role in modulating microtubule dynamics, we next asked whether survivin influenced microtubule stability in fixed cell preparations. We expressed GFP-tagged survivin in COS-7 cells using a replication-deficient adenovirus (GFP-survivin) (Mesri *et al.*, 2001) and analyzed cultures by fluorescence microscopy for acetylated tubulin (see above). Interphase cells expressing GFP-survivin showed an increase in the amount of acetylated tubulin compared with GFP-expressing control cells (Figure 6, A–D). In addition, cells in cytokinesis showed an increase in the amount of actylated tubulin at midbodies when survivin levels were increased (Figure 6, E–H). Moreover, survivin-expressing interphase cells treated with the microtubule-depolymerizing agent nocodazole showed increased resistance to microtubule depolymerization compared with controls (Figure 6K). Twenty minutes after nocodazole treatment, most microtubules were depolymerized in control cells (Figure 6I, GFP), whereas microtubules persisted in survivin-expressing cells at this time (Figure 6J) and for an additional 40 min.

Depletion or Pharmacologic Inhibition of Aurora B Kinase Does Not Affect Microtubule Dynamics or Nucleation

To investigate the mechanism by which survivin modulates microtubule dynamics and nucleation, we first tested whether Aurora B perturbed microtubule dynamics when depleted by RNAi. Aurora B depletion was achieved using siRNAs previously used in studies to deplete Aurora B (Hauf *et al.*, 2003). These effectively reduced Aurora B levels in RPE cells by 60–80% (Figure 7A). Reduction of Aurora B expression by siRNA was associated with formation of binucleated cells presumably because of cytokinesis failure (Figure 7, B and C), in agreement with published results. However, analysis of microtubule stability using acetylated tubulin antibodies under these experimental conditions revealed no significant differences between control and Aurora B siRNA-treated cultures (Figure 7, D–F). To formally test whether Aurora B suppression by siRNA affected microtubule dynamics, we used time-lapse imaging of stably transfected cells expressing GFP-labeled α-tubulin. In these experiments, the frequency of microtubule rescue and catastrophe, the duration of microtubule pause, growth and shrinking, and the rate of growth and shrinking were indistinguishable from cultures treated with control (VIII) or Aurora B-directed siRNA (Figure 8, A and B, and Table 1). To independently validate these results, we used time-lapse imaging of microtubule growth in living cells expressing GFP-EB1. In these experiments, Aurora B suppression by siRNA did not significantly alter microtubule growth distances and the number of GFP-EB1 foci, compared with control (VIII)-transfected cells (Figure 8, C and D). We also showed that immunoprecipitation of survivin from logarithmically growing or mitotic HeLa cells did not pull down detectable Aurora B, although the survivin-binding protein heat-shock protein of 90 kDa (Hsp90) effectively coimmunoprecipitated with survivin (Figure 8E, cells overexpressing survivin did not pull down detectable levels of Aurora B; our unpublished data). These biochemical experiments suggest that at least some sur-
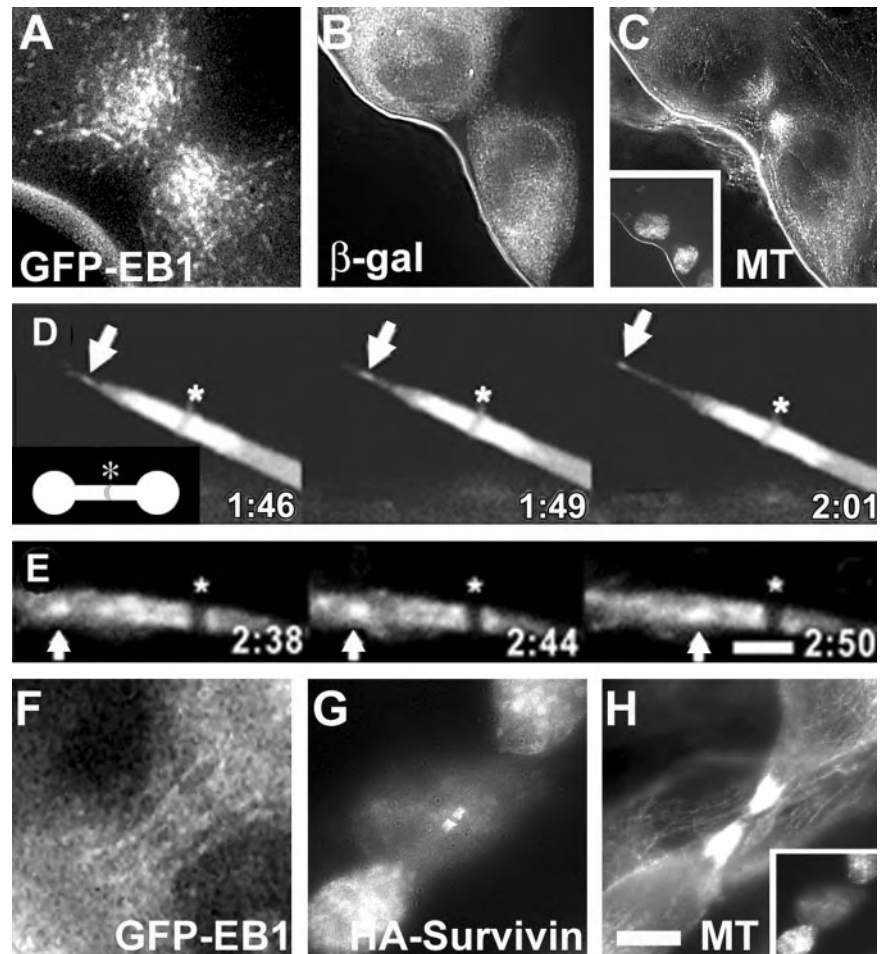
Figure 5. Increased levels of survivin suppress microtubule growth in midbodies during cytokinesis. (A–C) GFP-EB1 staining at midbodies in telophase cells expressing control protein (β-galactosidase) or survivin (F–H). Midbody GFP-EB1 labeling is significant in control cells (A–C), with movements (representing microtubule growth) both away from the central midbody region (D; arrow) and toward the central midbody (E; arrow) (see Movies 8 and 9). Schematic in D shows midbody region examined in this figure. In survivin-expressing cells (F–H), little GFP-EB1 labeling is observed at the midbody (F) although the cell is at a similar stage of cytokinesis to that in A. Insets (A–C and F–H), DNA labeled with DAPI. Images in A and F represent enlargements of midbodies seen in B and G, respectively. Bar (H), 10 μm for B, C, G, and H and 5 μm for A and F. Bar (E), 5 μm for D and E.

vivin was not bound to Aurora B kinase in HeLa cells under these conditions, a result different from studies done in *Xenopus* extracts or cells ectopically expressing the proteins (see *Discussion*) (Bolton *et al.*, 2002; Beardmore *et al.*, 2004, Temme *et al.*, 2005).

Because recent data suggests that depletion of Aurora B can affect the cellular levels of survivin and vice versa (Honda *et al.*, 2003), we used the pharmacologic agent hesperadin to inhibit the activity of the kinase as done previously (Hauf *et al.*, 2003). Treatment with hesperadin dramatically reduced phosphorylation of the Aurora B target protein histone H3 compared with cells exposed to an inactive hesperadin analogue (Figure 9A). Hesperadin induced defects in spindle organization and chromosome alignment (17/17 spindles and 0/10 controls; Figure 9, B–D). Spindles were usually more narrow and sometimes longer than controls, and the two halves of the spindles were misoriented in that they were not aligned 180° from one another but crescent shaped. Chromosomes were often positioned outside the area occupied by spindle microtubules (Figure 9, C and D). The spindle defects in hesperadin-treated cells were distinct from those observed in survivin-expressing cells, where spindles were often shortened in the pole-to-pole dimension but had normally aligned chromosomes (Figure 4G) (Giodini *et al.*, 2002). The different spindle disruption phenotypes suggested that Aurora B and survivin affected spindles by different mechanisms, providing additional support for the idea that these proteins functioned independently and not as members of a common protein complex.

Cells treated with either hesperadin or the inactive analogue showed no differences in the rates of microtubule growth or shrinking, the duration of microtubule growth, shrinking or pause, the frequency of microtubule catastrophe, or rescue as collectively measured by time-lapse and fixed cell imaging of GFP-α tubulin or GFP-EB1 (Figure 9, B–F, and Table 1). Moreover, hesperadin treatment of survivin-depleted cells showed no effect on microtubule nucleation, suggesting that there was no contribution of Aurora B in the context of reduced survivin (Figure 3C). This was consistent with our data showing that survivin depletion did not significantly affect Aurora B levels and vice versa (Figure 7A; see *Discussion*). Together with data from the Aurora B depletion experiments, these results show that inhibition of Aurora B activity or levels has no effect on microtubule dynamics despite induction of dramatic defects in mitosis under both conditions. The microtubule dynamics and nucleation changes seen in cells with altered survivin levels seem to be independent of Aurora B and could be induced by a fraction of survivin that is not associated with the chromosomal passenger complex or by survivin within the complex.

DISCUSSION

In this study, we have shown that survivin functions as a novel regulator of microtubule dynamics and microtubule nucleation throughout the cell cycle and that this pathway is independent of the expression or activity of the chromosomal passenger protein Aurora B. Time-lapse imaging of living cells using two
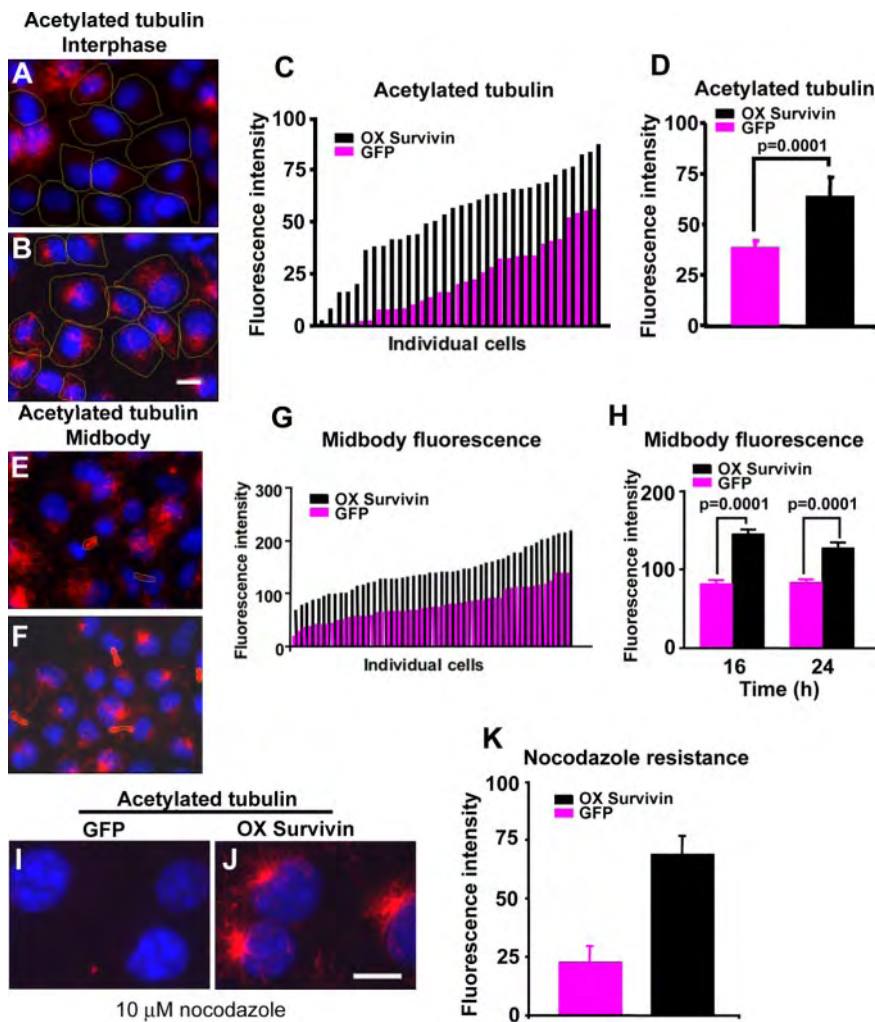
Figure 6. Increased levels of survivin increase microtubule stability. (A and B) Survivin increases acetylated tubulin content. COS-7 cells transduced by pAd-GFP (A) or pAd-GFP-survivin (B) were stained by immunofluorescence using an antibody to a stabilized acetylated form of α-tubulin. Bar (B), 5 μm for A and B. Individual cells outlined. (C and D) Quantification of acetylated tubulin signal in individual cells (C; total integrated fluorescence of a single representative experiment) and in all cells (D; expressed as an average, n > 2 × 10³ measurements from optical sections taken from >200 cells/bar). All data for A–D were acquired from interphase cells). (E and F) Survivin increases acetylated microtubules at midbodies. Cells were transduced with pAd-GFP (E) or pAd-GFP-survivin (F) and analyzed with an antibody to acetylated tubulin by fluorescence microscopy. Bar (F), 5 μm for E and F. (G and H) Quantification of acetylated tubulin signal at midbodies in individual cells (G) or whole cell population (H). (I and J) Nocodazole resistance. Cells were transduced with pAd-GFP or pAd-GFP-survivin, exposed to nocodazole for 20 min (I and J), and analyzed for α-tubulin staining by fluorescence microscopy. I and J represent high-magnification images of cells acquired from random fields for analysis. Bar (J), 5 μm for I and J. (K) Quantification of nocodazole resistance of microtubules (α-tubulin staining) in cells expressing GFP or survivin at 20 min. Fluorescence intensity is in arbitrary units.

independent GFP-labeled microtubule markers, the plus-end protein EB1 and α-tubulin, combined with quantitative analysis of multiple parameters of microtubule dynamics revealed that survivin affected rates of microtubule catastrophe and the degree of centrosomal microtubule nucleation.

Our results are consistent with a role for survivin in microtubule dynamics and microtubule nucleation independent of at least one protein of the chromosomal passenger complex (Aurora B) for several reasons. First, we found no effect on microtubule dynamics or nucleation when Aurora B was pharmacologically inactivated during a short incubation periods (up to 6 h), even though inhibitory activity dramatically affected spindle function. Second, inhibition of Aurora B activity or depletion of Aurora B levels produced spindle phenotypes dramatically different from survivin-depleted or -overexpressing cells. Third, survivin affected microtubule dynamics and nucleation in interphase when Aurora B is thought to be absent or drastically reduced. Fourth, our previous studies show that survivin is in multiple separate compartments within cells (Fortugno *et al.*, 2002), whereas Aurora B and other chromosomal passenger proteins have not been localized to these other sites. Fifth, our biochemical studies suggest that cells possess a fraction of survivin that is not physically associated with the chromosomal passenger complex and even if it was in the complex, survivin could still affect microtubule dynamics and nucleation independent of other members of the complex.

A Unifying Model for the Multiplicity of Survivin Phenotypes?

The work in this manuscript provides new mechanistic insights into the complex functions of survivin at multiple cell cycle stages. We propose a model in which survivin modulation of microtubule dynamics and nucleation contributes to microtubule-based functions at multiple cell cycle stages and at all cellular sites to which the protein is localized. In this model, survivin could modulate the organization and/or function of mitotic spindles (Li *et al.*, 1999; Giodini *et al.*, 2002; Okada *et al.*, 2004), the spindle checkpoint (Carvalho *et al.*, 2003; Lens *et al.*, 2003), midbody activities (Adams *et al.*, 2001), centrosome-mediated microtubule nucleation and organization (Li *et al.*, 1999), and interphase microtubule-based processes.

Two Pathways to Regulate Microtubule Dynamics

The mitotic function of survivin is thought to be related to its localization to kinetochores, and in particular its association with at least some "chromosomal passenger proteins" (Wheatley *et al.*, 2001). In addition to its potential involvement in proper kinetochore attachment, central spindle formation, and cytokinesis, the chromosomal passenger complex has been more recently implicated in a Ran-GTP-independent pathway of bipolar spindle assembly via Aurora B inhibitory phosphorylation of the microtubule-depolymerizing activity of the Kin I kinesin MCAK. It remains possible that survivin indirectly
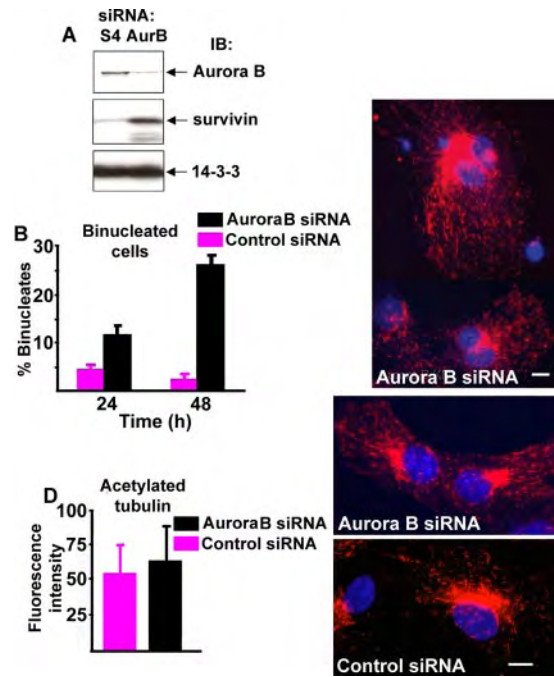
Figure 7. Aurora B silencing has no detectable effect on microtubule dynamics. (A) RPE cells were transfected with a survivin-specific siRNA or an Aurora B-specific siRNA, harvested 48 h later, and analyzed by immunoblotting. (B) Binucleated cells were quantified 24 and 48 h after transfection of Aurora B and control siRNAs (n = 300 cells/bar). (C) Image showing two binucleated cells after treatment with Aurora B siRNA (arrows). Nuclei, blue, microtubules, red. (D) Quantification of microtubule acetylation following Aurora B or control siRNA treatment (n = 200 cells for each bar). (E and F) Images of acetylated microtubules in cells treated with Aurora B (E) or control (F) siRNAs. Nuclei, blue, acetylated microtubules, red. Bar (C), 5 μm; bar (F), 5 μm for E and F.

regulates MCAK activity, and therefore microtubule stability, at the kinetochore. However, our data argue for the existence of a separate, survivin-selective/specific pathway for modulating microtubule dynamics. For example, the complex could be selectively involved in modulating microtubule stability at kinetochores, whereas survivin could participate in more global mechanisms of spindle assembly/function, midbody function and interphase microtubule nucleation/organization. The differences in the ability to detect an interaction of Aurora B and survivin observed by different investigators will require further investigation. It is possible that these differences reflect an interaction that is labile or transient. In any case, we think that the ability of survivin to modulate microtubule nucleation and dynamics in an Aurora B-independent manner could be achieved whether the protein is alone or in a complex with Aurora B and other members of the chromosomal passenger complex.

Survivin as a Dual-Function Protein

The IAP gene family is comprised of two classes of molecules, one implicated in cell division and a second that suppresses apoptosis via inhibition of caspase maturation and/or activity. However, as a structurally unique IAP family member, survivin is an apparent exception to this rigid definition, and accumulating evidence indicates a role for the protein in both functions (Altieri, 2003). Survivin seems to inhibit apoptosis through a pathway centered on intermolecular cooperation
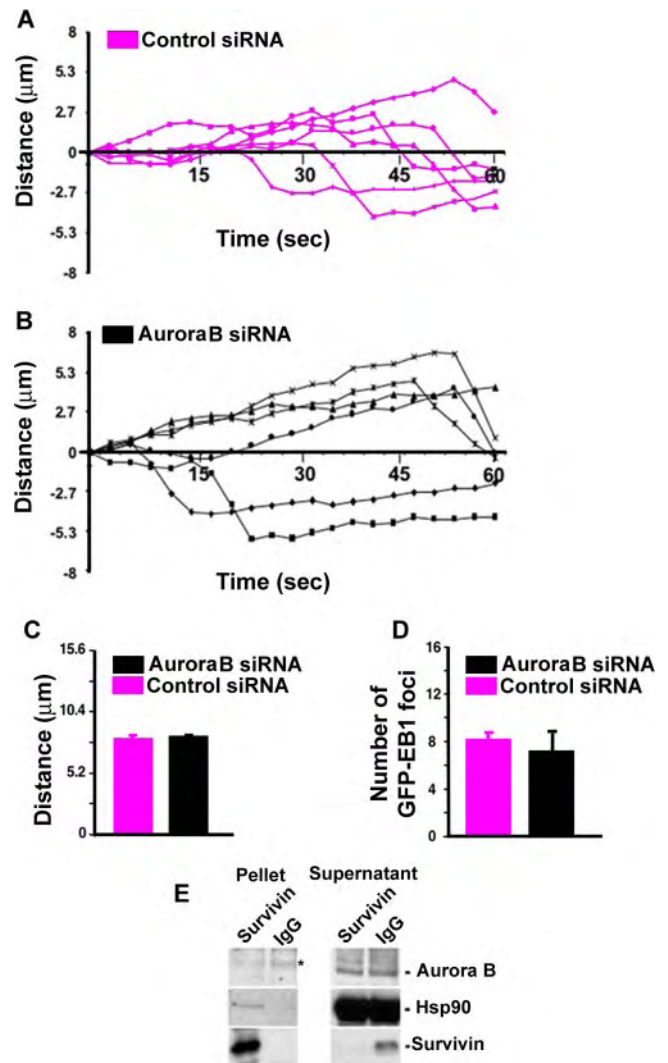
Figure 8. Aurora B silencing has no detectable effect on microtubule dynamics. (A and B) RPE cells stably expressing GFP α-tubulin were transfected with Aurora B (B) or control (A; VIII) siRNAs. Images of microtubules in interphase cells were acquired every 3 s, and the resulting time-lapse movies were used to construct history plots of individual microtubules. No significant differences were observed in microtubule catastrophe or rescue frequencies (6 microtubules from 2 cells are shown), the duration of microtubule pause, growth or shrinking, or the rate of microtubule growth or shrinking (see Table 1). (C and D) Silencing of Aurora B has no detectable effect on the distance traveled for GFP-EB1 foci (C; tracking distances) or the number of GFP-EB1 foci per unit area (D) (n = 16 microtubules from 3 or more cells for each bar). See legends to Figures 1 and 2 and Table 1 for more details. (E) Survivin was immunoprecipitated from asynchronous cultures of HeLa cells using previously characterized antibodies (Giodini *et al.*, 2002) and probed for survivin, Hsp90 and Aurora B as indicated. Although Aurora B can be detected in the cell lysates it is undetectable in the survivin immunoprecipitation. IgG is nonimmune rabbit IgG used for immunoprecipitation control. Asterisk (*) represents the IgG band.

with cofactors (Marusawa *et al.*, 2003) and dynamic subcellular shuttling of a mitochondrial pool of survivin. The role of survivin in mitotic control has remained controversial. Although survivin is unanimously viewed as indispensable for cell division, given the panoply of catastrophic mitotic defects induced by functional abrogation of the protein, the mechanism of
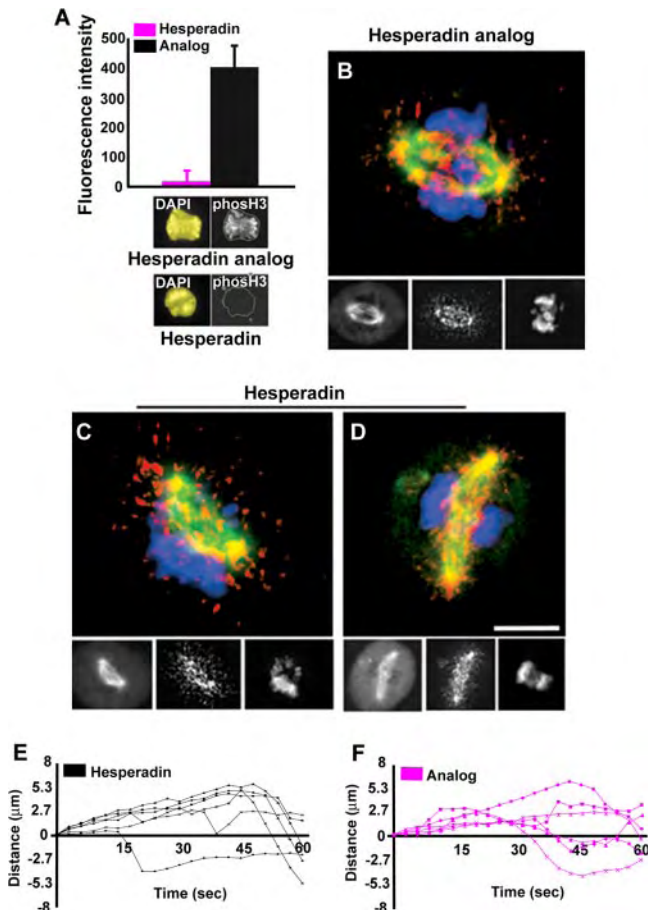
Figure 9. Chemical inhibition of Aurora B activity by hesperadin does not affect microtubule dynamics in interphase or mitotic cells. (A) RPE cells treated with 100 nM hesperadin or a nonfunctional analog for 6 h were used to quantify the phosphorylation of the Aurora B target, histone H3 (A; phos-H3). Graph, n = 10 randomly chosen mitotic cells/bar. Representative images are shown below bars. DAPI panel shows DNA pattern, and the phos-H3 panel shows staining with antibody specific for phosphorylated H3. DAPI staining was used to identify regions occupied by chromatin given the drastic loss of phos-H3 in hesperadin-treated cells. (B–D) Mitotic spindles in hesperidin-treated cells are narrower (C) and angled (D) compared with controls (B). Microtubule growth as measured by the distribution of GFP-EB1 foci (red) in mitotic cells treated with hesperadin (C and D) or the control analog (B) show no significant differences. Large images represent merge of microtubules (green), GFP-EB1 (red), and DNA (DAPI; blue). Smaller images below show, from left to right, α-tubulin staining (microtubules), GFP-EB1, and DNA. Bar (D), 5 μm for B–D. (E and F) Microtubule dynamics in interphase cells treated with hesperadin (E) or the analogue (F) show no significant differences (6 microtubules from 2 cells shown; also see Table 1).

mitotic disruption and the cell cycle stages regulated by this protein are unclear. Our results suggest that the complex series of interphase and mitotic defects induced by changes in survivin levels are modulated through its ability to control microtubule nucleation and dynamics.

Survivin-dependent Changes in Microtubule Dynamics during Cytokinesis

Our data demonstrate a role for survivin in cytokinesis, which was originally proposed by analogy with ancestral IAP proteins in model organisms (Speliotes *et al.*, 2000) and by obser-

vations linking survivin depletion to regression of cleavage furrow (Chen *et al.*, 2000). In this study, we first show that midbody microtubules are highly dynamic in normal cells, a surprising result given the apparent static nature and high stability of this population of microtubules (high level of acetylated tubulin). Moreover, we show that microtubules grow toward the central midbody as might be expected, but also away from the midbody. This suggests the presence of several microtubule populations at this site. They could arise from overlapping microtubules of the central spindle (plus ends at center), cytoplasmic microtubules that subsequently invade the midbody, or midbody-generated microtubules possibly nucleated by γ-tubulin at the central midbody. Further studies will be required to elucidate the functions of these populations of midbody microtubules. The dynamics of these microtubule populations is severely inhibited when survivin levels are altered, and this may account for the observed cytokinesis defects.

Survivin-dependent Changes in Microtubule Dynamics in Interphase

An important conclusion of this study is that the effect of survivin depletion or overexpression on microtubule dynamics was not restricted to mitosis but occurred throughout multiple cell cycle phases, including interphase. There is already convincing evidence that survivin expression may be induced outside mitosis, and this has been experimentally validated for cytokine-stimulated hematopoietic progenitors, angiogenic endothelial cells, and tumor cells where survivin is abundantly overexpressed at all cell cycle phases (Altieri, 2003). This may reflect distinct transcriptional mechanisms of survivin gene expression at different cell cycle phases, as exemplified by the recently reported role of E2F family proteins at inducing survivin expression at the G_1/S transition. Accordingly, the ability of survivin to control microtubule dynamics at multiple cell cycle phases may have dramatic repercussions for cancer cells, promoting aneuploidy at cell division but also potentially altering cell polarity through disruption of microtubule dynamics and nucleation in interphase. Moreover, the interphase effects of survivin on microtubules provide additional evidence for an Aurora B-independent activity because the kinase is thought to be mitosis specific.

In summary, our data demonstrate that survivin functions in a continuum throughout multiple cell cycle phases and its role is centered on the regulation of microtubule dynamics and nucleation. The independence of this pathway from Aurora B kinase expression and activity suggests that it may provide a novel mechanism of microtubule regulation in both mitotic and interphase cells, and a potential critical point of intervention for molecular antagonists of survivin as rational anticancer agents (Altieri, 2003). The overexpression of survivin in nearly every human tumor underlines the importance of this endeavor.

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A role for IKK in centrosome duplication and regulation of mitosis

Sven Rottenberg, [1] Keith Mikule, [2] Rebekka Schwab, [1]*Volker T. Heussler, [1§] Paula C.

Fernandez, [1] Florian Virchow, [1] Stephen Doxsey,[2] Dirk A. E. Dobbelaere[1].

[1]Division of Molecular Pathology, ITPA, University of Bern, CH-3012 Bern Switzerland

[2]Department of Molecular Medicine, University of Massachusetts Medical School,

Worcester, Massachusetts 01605

*present address: Molecular Haematology and Cancer Biology Unit, Institute of Child

Health, UCL, London, UK

§present address: Berhard-Nocht-Institute for Tropical Medicine, D-20359 Hamburg,

Germany

**IKK is best known as a central regulator of NF-κB-dependent transcription that is
activated in response to a range of environmental signals. We now identify IKK as an
integral component of the centrosome, where it is localized throughout the cell cycle
and activated during G2 and M. Subsequently, IKK appears in its active form at the**

midbody during cytokinesis. Downregulation of IKK inhibited centrosome duplication and promoted cytokinesis failure and the generation of multinucleate cells. Blocking IKK in prometaphase cells prevented progression through mitosis in a transcription-independent manner. Taken together, we demonstrate important new roles for IKK in regulating centrosome function and cell cycle progression.

Centrosomes are the major microtubule-organizing centers in eukaryotic cells and are required for successful completion of the cell cycle (*1, 2*). While the role of centrosome-associated regulatory activities is not completely understood, it has been suggested that centrosomes provide a central site for coupling both intracellular and extracellular signals to cellular function (*2, 3*). Centrosome dysfunction can induce mitotic spindle defects or cytokinesis failure resulting in aneuploidy, features that are shared by tumor cells (*4*). The centrosome duplicates once during every cell cycle and the resulting pair of centrosomes organizes the bipolar spindle. Defects in the duplication process can lead to disorganized spindles and mitotic failure. Moreover, removal of centrosomes from cells or reducing the levels of centrosome proteins causes cells to arrest in G1 prior to DNA synthesis, thus preventing additional rounds of centrosome duplication (*5-8*). Centrosome duplication shares many features with DNA replication. Both processes occur coincidently, are semi-conservative and require cyclin-dependent kinase 2 (Cdk2) and S-phase cyclins (*9, 10*). Recent evidence demonstrates that centrosomes serve as docking sites for a growing number of regulatory molecules that control centrosome-associated processes, as well as global cellular processes such as cell cycle progression (*5, 7*). The use of common

regulatory complexes to control centrosome, DNA and cell cycles appears to be one way of coupling all three cycles to ensure faithful segregation of chromosomes during cell division. Localization of cell cycle regulatory complexes to centrosomes may facilitate coordinating these processes. However, the precise mechanism by which the three cycles are connected to one another and how they respond to extracellular cues are still largely unknown.

The NF-κB activation pathway is best known for its role as a central regulator of inflammatory and immune responses, but it also contributes to developmental processes, protection against programmed cell death and cell-cycle regulation (*11-13*). Consequently, NF-κB has increasingly become linked to cancer (*14*). NF-κB activation pathways converge onto a multi-subunit kinase complex, the IKK (IκB kinase) complex, that typically consists of two catalytic subunits, IKK1 (IKKα) and IKK2 (IKKβ), and a modulating anchoring unit NEMO (IKKγ). Activated IKK phosphorylates IκBs, the cytoplasmic inhibitors of NF-κB, tagging them for polyubiquitination and rapid proteasomal degradation, allowing released NF-κB to translocate to the nucleus. The spectrum of IKK substrates was recently extended by the finding that IKK1 contributes to transcriptional regulation by phosphorylating histone H3 (*15, 16*). IKK activation can be induced by recruiting the complex to specific cellular sites where activating triggers are delivered. These include plasma membrane receptors such as the TNF receptor (*17, 18*)and the T cell receptor (*19, 20*) as well as the surface of the intracytoplasmic parasite *Theileria* (*21*). These findings suggest that the intracellular topology of IKK activation may determine the specificity of the activated pathway as well as its biological outcome.

While studying IKK recruitment to the intracellular pathogen *Theileria* (*21*), we noticed that IKK consistently localized to host cell centrosomes. Using a panel of antibodies directed against IKK1, IKK2, and NEMO, combined with a centrosome-specific marker (anti-γ-tubulin), we confirmed the existence of centrosomal IKK complexes (Fig. 1A-E and fig. S1A-C). IKK association with centrosomes is a general phenomenon that was found in all cell lines tested, including Jurkat T-cells, COS-7, U2OS, human retinal pigment epithelial (hTERT-RPE-1), HeLa as well as in primary peripheral blood mononuclear cells. Moreover, ectopically expressed V5-epitope-tagged forms of IKK1, IKK2 or NEMO all localized to centrosomes (shown for IKK1 in fig. S2). IKK appears to be an integral centrosome component as its centrosomal localization was unaltered when microtubules were depolymerized with nocodazole (fig. S1D).

Each centrosome is comprised of two centrioles (one maternal centriole and one daughter derived from the mother during the previous cell cycle) and the surrounding pericentriolar material. The maternal centriole anchors the microtubule aster and is distinguished by the presence of appendages that contain several proteins including centriolin (*7*) and ninein (*2, 22*). Double immunofluorescence experiments in COS-7 cells revealed that ectopically expressed V5-epitope-tagged IKK1 colocalized to the maternal centriole with centriolin (fig. S3) and ninein (not shown). NEMO also localized to the maternal centriole at the base of the primary cilium following exposure of RPE-1 cells to low serum (fig. S3). During cytokinesis, IKK1 was found to localize centrally in the mid-body (Fig. 1F) as reported for other midbody proteins (*23*) while IKK2 flanked the central mid-body staining of IKK1 and colocalized with the passenger protein AIM-1(*24*).

NEMO has been shown to function both as a regulator of IKK activity and a scaffolding protein for the complex (*12*). To test whether NEMO serves as a scaffold for centrosomal IKK, we silenced NEMO and examined the localization of the two IKK components. Treatment with siNEMO resulted in a ~50% reduction in the total cellular levels of the protein (Fig. 2A, inset) as well as a corresponding decrease in centrosome-associated NEMO, IKK1 and IKK2 (Fig. 2A). Centrosomal γ-tubulin on the other hand was not affected. These findings demonstrate that NEMO anchors IKK to centrosomes.

Localization of IKK to centrosomes suggested a role in centrosome function or cell cycle progression. Because the best know role of centrosomes is the organization of microtubules, we first examined interphase microtubule arrays, mitotic spindles and centrosome-mediated microtubule nucleation in cells silenced for IKK. However, no significant changes were detected in these parameters. We next examined the effect of IKK silencing on centrosome duplication. U2OS cells were treated with hydroxyurea (HU) to induce S-phase arrest, a condition permissive for multiple rounds of centrosome duplication in the absence of DNA replication (*25, 26*). Centrosome amplification (more than two centrosomes per cell) was observed in control cells treated with siRNAs targeting the control protein lamin, but was reduced in cells treated with siNEMO (Fig. 2B). This suggested a role for IKK in centrosome duplication. To address this issue more directly, we examined centrosome duplication in HeLa cells that progressed normally through the cell cycle (in the absence of HU-induced S-phase arrest). NEMO silencing caused a progressive loss of centrioles from spindle poles in mitosis (Fig. 2C), a result similar to that observed upon silencing of the centrosome duplication gene centrin-2 (fig. S4) (*27*). In

addition, NEMO silencing induced formation of binucleate and multinucleate cells resulting from cytokinesis failure (Fig. 2D and fig. S4) as seen in cells with reduced centrin-2 (*27*) or centriolin (*7*) or in cells with increased levels of the centrosomal aurora A kinase (*28*).

While IKK was found associated with the centrosome throughout the cell cycle, activation of centrosomal IKK was cell cycle-regulated. Immunofluorescence analysis using antibodies that recognize activation-specific phosphorylation of IKK1 and IKK2 (P-IKK) revealed pronounced centrosomal staining particularly in G2 and during mitosis. Quantitative analysis showed a steady increase in centrosomal P-IKK during this period reaching maximal intensity in prometaphase and metaphase cells (Fig. 3A and B). In addition to P-IKK, phosphorylated IκBα (P-IκBα) was also detected at centrosomes and displayed an identical staining pattern to P-IKK (Fig. 3C). Activated IKK was also localized to multiple centrosomes in cells with multipolar spindles (Fig. 3D), which have been shown to missegragate chromosomes and lead to aneuploidy and possibly cancer (*4, 29, 30*).

As cells exited mitosis and entered cytokinesis, centrosomal levels of P-IKK and P-IκBα decreased and both appeared at the midbody (Fig. 3D) as originally seen with IKK1 (see Fig. 1F). These results demonstrate that the activated forms of IKK and its substrate IκBα localize to centrosomes and midbodies, intracellular structures whose functions are perturbed upon disruption of the kinase complex.

At the G2 to M transition, when IKK is activated at the centrosomes, several events take place that are dependent on cyclin B-Cdk1 activity. To determine whether IKK

activation at centrosomes required cyclin B-Cdk activity, we used to Cdk inhibitor butyrolactone I (*31*). In COS-7 cells treated with butyrolactone I during G2 to M transition, the level of centrosomal IKK phosphorylation was reduced by more than 50 % compared to untreated cells (Fig 3F). This demonstrated that cyclin B-dependent kinase activity contributes to centrosomal IKK activation during this cell cycle stage.

During the G1 phase of the cell cycle, centrosomal IKK is not activated and centrosome-associated IκBα not phosphorylated. To address whether centrosomal IKK can be activated by triggering the NF-κB activation pathway, we stimulated COS-7 cells with the phorbol ester PMA. PMA did not induce the activation of centrosomal IKK and phosphorylation of centrosomal IκBα, even though it triggered the robust phosphorylation and degradation of cytosolic IκBα as expected (data not shown). This indicates that conventional IKK activation does not extend to centrosomal IKK. Rather, centrosomal IKK is activated in a cell cycle-dependent manner during G2/M.

Centrosomes have recently been shown to play a role in cell cycle progression (*6-8*). The fact that centrosomal IKK becomes increasingly activated as cells proceed through G2 and enter M, suggested a role for IKK in the progression through mitosis. To investigate this, COS-7 cells were arrested in mitosis by nocodazole treatment, collected by mitotic shake-off and subsequently cultured in the presence or absence of IKK inhibitors. Flow-cytometric analysis showed that within 2h of release from nocodazole block, 48 % of control cells had completed mitosis/cytokinesis and entered G1 (4). The presence of IKK inhibitors such as sulfasalazine, prostaglandin A2 or prostaglandin J2, markedly reduced progression from prometaphase into G1. As IKK is a key regulator of NF-κB-dependent

transcription, we addressed the question whether activation of centrosomal IKK during mitosis is linked to transcription. Although transcription is largely downregulated as cells enter mitosis, selective RNA polymerase II-dependent transcription has been demonstrated in cells undergoing mitosis (*32*). In the presence of the general transcription blocker actinomycin D, similar numbers of cells progressed from prometaphase to G1 as in control cultures (4), indicating that de novo transcription - including NF-κB-dependent transcription - is not required at this stage. This strongly suggests that the function of activated centrosomal IKK in prometaphase to G1 transition is not linked to NF-κB-dependent transcription.

Cytoplasmic IKK has been shown to undergo translocation in response to signaling, (*15-20*), but a permanent association with a cellular structure has not been demonstrated before. The finding that the entire IKK complex is an integral member of the family of centrosomal proteins is surprising, in particular as it becomes phosphorylated and activated in a cell cycle-dependent manner during G2 and M, and when it appears at the midbody during cytokinesis. A range of regulatory proteins localize to the centrosome during the cell cycle (see reviews by (*2*) and (*22*) and references therein). Large coiled-coil proteins such as c-Nap1 (*33*), AKAP450, pericentrin (*34*) and centriolin (*7*) associate with the maternal centriole where they appear to anchor regulatory components. NEMO, a stochiometric component of the IKK complex (*35*), is also a coiled-coil protein and is required for tethering the catalytic IKK subunits to the centrosome. Whether NEMO anchors proteins with regulatory function other than IKK is presently unknown.

The fact that interference with IKK function inhibits centrosome duplication and results in failure to complete mitosis and cytokinesis, highlights the importance of this kinase in the regulation of centrosome function and the cell cycle. The control exerted by IKK on these events may require the concerted action of both cytoplasmic and centrosomal IKK, and involve transcription-dependent as well as -independent mechanisms. By regulating NF-κB-dependent cyclin gene expression, IKK contributes to the activation of cdks, Rb phosphorylation and activation of the transcription factor E2F. Activated E2F not only controls the expression of genes required for entry into S and DNA replication (*13*), but also regulates centrosome duplication (*9, 10, 36*). Consistent with these observations, we found that siRNA-mediated downregulation of NEMO, which blocked centrosome duplication in U2OS and HeLa cells, also inhibited DNA replication in telomerase-immortalized RPE-1 cells (K.M. and S.R. unpublished). IKK-mediated activation of NF-κB-dependent cyclin gene expression may thus contribute to both processes. Nevertheless, as an integral centrosomal component, IKK might also contribute directly to centrosome duplication. This is suggested by the fact that downregulation of NEMO interfered with centrosome duplication in Rb-deficient U2OS cells in which E2F is constitutively active.

Inhibiting IKK blocks progression from prometaphase to G1, a process that does not require transcription. The fact that IKK can function as a regulator of mitosis in a transcription-independent manner is intriguing. The gradual increase in IKK phosphorylation during G2/M and the absence of centrosomal IKK phosphorylation in response to PMA-induced activation of the classical NF-κB pathway, both argue against an on/off switch that is linked to the general NF-κB activation machinery. Full activation may

be determined by the relative activities of kinases and/or phosphatases in a time-dependent manner and be subject to different cell cycle-dependent signals, potentially involving the cyclin B/cdk1 complex, that converge onto centrosomal IKK. In this context, it is interesting to note that mixed-lineage kinase 3 (*37*), which has been associated with IKK activation (*38*), also localizes to the centrosome, but a link to cell cycle regulation or centrosome function was not established.

IκBα, the classical substrate of IKK, is found at the centrosome and midbody, and is phosphorylated whenever activated IKK is detectable. In line with the centrosome's role as an organizing centre where enzymes and substrates are brought together, components of the SCF ubiquitin ligase complex as well as proteasomal machinery have been found in association with centrosomes (*39*). This could allow IκB ubiquitination and degradation of P-IκBα at the centrosome to occur, resulting in the release of a pool of free NF-κB, which, while not being required for mitosis/cytokinesis, is immediately available to participate in NF-κB-dependent gene expression in the ensuing G1 phase. However, considering the fact that IKK1 has recently been shown to phosphorylate histone H3 (*15, 16*), it cannot be excluded that centrosomal IKK phosphorylates proteins other than IκBα. Alternatively, phosphorylation of IκBα by centrosomal IKK might trigger the release of proteins, other than NF-κB, that regulate progression through mitosis.

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Figure legends

Fig. 1. The IKK complex localizes to centrosomes throughout the cell cycle and is
associated with the midbody during cytokinesis. **(A)** Immunofluorescence microscopy
analysis showing the association of IKK (detected by anti-IKK1, green) with centrosomes
(detected by anti-γ-tubulin, red) in an interphase cells in G1. **(B)** In a minor population of
interphase cells containing two centrosomes (γ-tubulin, red), IKK (green) localizes to only
one centrosome (see also fig. S3). **(C)** Interphase cell (G2) showing IKK1 associated with
both centrosomes. **(D)** Cell in prophase. (E) Mitotic cell in metaphase. **(F)** IKK1 (green)
localizes to the central region of the midbody (labeled with anti-AIM-1, red). **(G)** IKK2
(green) colocalizes with AIM-1 (red). "DNA" indicates staining of the nuclei with Hoechst
DNA dye. "Merge" represents an overlay of the two images. COS-7 cells were used for A,
B, C, and F; HeLa cells for D and E.

Fig. 2. Silencing of NEMO by siRNA blocks IKK recruitment to the centrosome, causes
centrosome duplication defects and results in the generation of multinucleate cells. **(A)**
Treatment of U2OS cells with siNEMO downregulates steady state levels of NEMO

(shown in inset by immunoblot analysis). The histogram shows a quantitative analysis of the average signal intensity of centrosomal NEMO, IKK1, IKK2 and γ-tubulin after NEMO knockdown (n ≥ 30 per bar, error bars indicate 95% CI). (**B**) Centrosome duplication was evaluated in U2OS cells after 64h of siRNA treatment; DNA replication was blocked by the addition of HU (4mM) for the last 40h in culture. Indirect immunofluorescence analysis was performed using anti-γ-tubulin to label the centrosomes and the percentage of cells containing more than 2 centrosomes determined; error bars indicate standard deviations for 3 independent experiments. (**C**) Centriole duplication monitored in HeLa cells (in the absence of HU-induced DNA replication arrest) treated with siNEMO for 48 h; metaphase cells lacking a pair of centrioles at each pole were scored as duplication-defective (error bars indicate standard deviations from 3 independent experiments). (**D**) Quantitative analysis of the number of nuclei per cell in cultures of HeLa cells (n=500) treated for 48 h with siNEMO or siControl.

Fig. 3. Activation and phosphorylation of centrosomal IKK during progression from G2 to M is accompanied by IκBα phosphorylation. During cytokinesis, P-IKK and P-IκBα are found at the midbody. (**A**) Levels of IKK phosphorylation increase as cells reach mitosis. COS-7 cells were stained with anti-P-IKK and the relative immunofluorescence signal intensity determined for individual centrosomes (n ≥ 30 per bar, error bars indicate a confidence interval of 95%). The diagram depicts the position of the centrosomes representative for each group of cells that was analyzed. (**B**) A COS-7 cell in mitosis, showing phosphorylated, centrosome-associated IKK (P-IKK, green) γ-tubulin is labeled

red; condensed chromosomes are blue), "Merge" represents an overlay of the two images.

(**C**) Centrosome-associated, phosphorylated IκBα (P-IκBα, green) in a COS-7 cell in

mitosis (γ-tubulin, red; condensed chromosomes, blue) (**D**) Merged image of P-IKK (green)

localizing to multiple centrosomes in a COS-7 cell with multipolar spindles (γ-tubulin, red);

chromosomes are stained blue. (**E**) During cytokinesis, P-IKK (green, top panel) and P-

IκBα (green, lower panel) both localize to the midbody (labeled using anti-AIM-1, red).

(**F**) Effect of the cdk inhibitor butyrolactone I on centrosomal IKK phosphorylation. COS-

7 cells were subjected to a double thymidine block and, 8 hours after release, incubated for

1h in the presence (+ but I) or absence (- but I) of 50μM butyrolactone I. The graph shows

the immunofluorescence signal intensity for individual centrosomes, obtained using anti-P-

IKK and anti-γ-tubulin for randomly selected butyrolactone I-treated cells, relative to

untreated cells (set at 100%); (n ≥ 30 cells per bar, error bars indicate the 95% CI).

Fig. 4. IKK Inhibitors interfere with the completion of mitosis in a transcription-

independent manner. Cells were synchronized in prometaphase by treatment with

nocodazole (0.1μg/ml, 16 h), collected by mitotic shake-off and cultured for 2h in the

absence (control) or presence of the IKK inhibitors sulfasalazine (sulfa), prostaglandin A1

(PGA1) or prostaglandin J2 (PGJ2). To determine whether progression from prometaphase

to G1 requires transcription, cells were also exposed to the transcription blocker

actinomycin D (Act. D). The histogram shows the percentage of cells in G1 determined by

flowcytometric analysis of their DNA content (error bars indicate the standard deviation).

Supplemental Figure 1
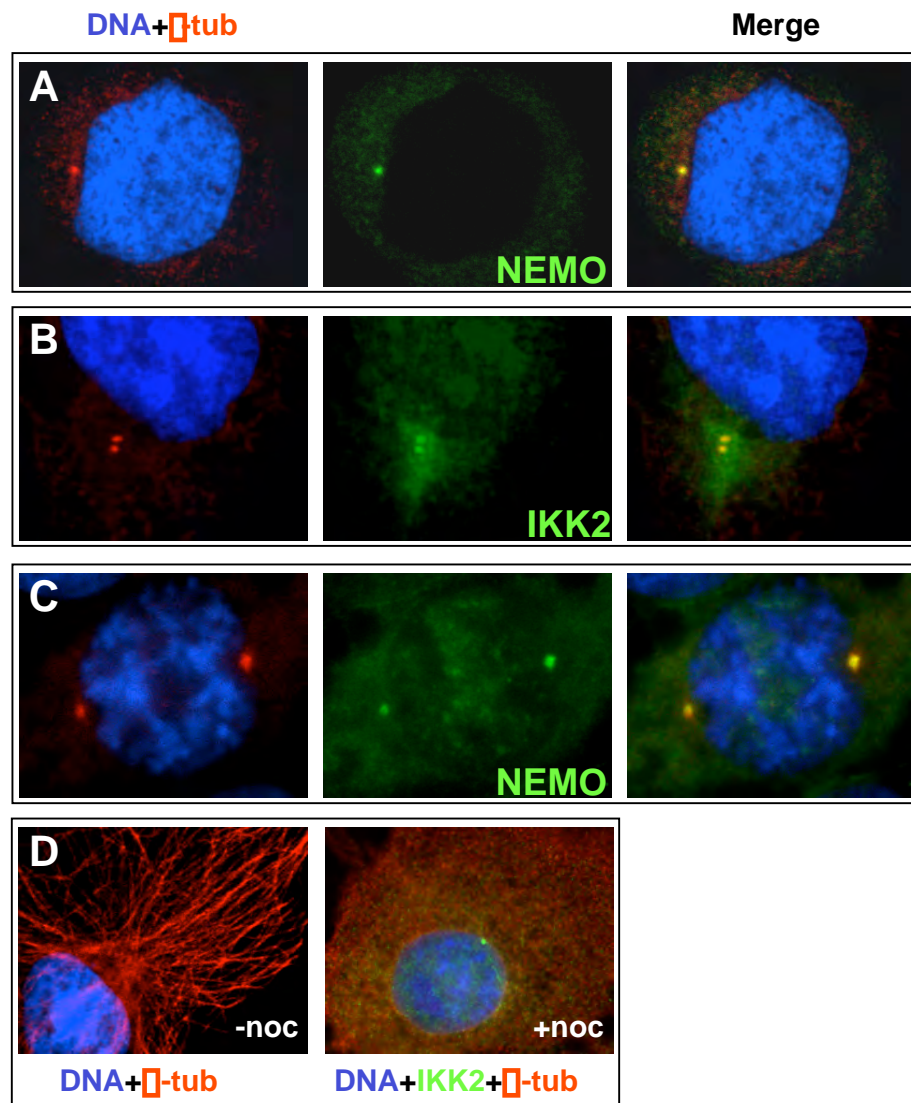
(A-C) IKK2 and NEMO also localize to the centrosome.
Jurkat T-cells **(A)**, COS-7 cells **(B)** or HeLa cells **(C)** were analyzed by immunofluorescence microscopy using anti-IKK2 or anti-NEMO antibodies (green) as indicated, together with anti-γ-tubulin (red); "DNA" indicates staining with Hoechst DNA dye (blue). "Merge" represents an overlay of the two images.
(D) IKK remains localized at the centrosome after nocodazole-induced microtubule depolymerization. Immunofluorescence analysis of COS-7 cells before (-noc) and after (+noc) depolymerization of microtubules induced by treatment with nocodazole (5 μg/ml) for 2 h. Microtubules are detected using an anti-α-tubulin antibody (red); anti-IKK2 was used to detect centrosomal IKK (green).

Supplemental Figure 2
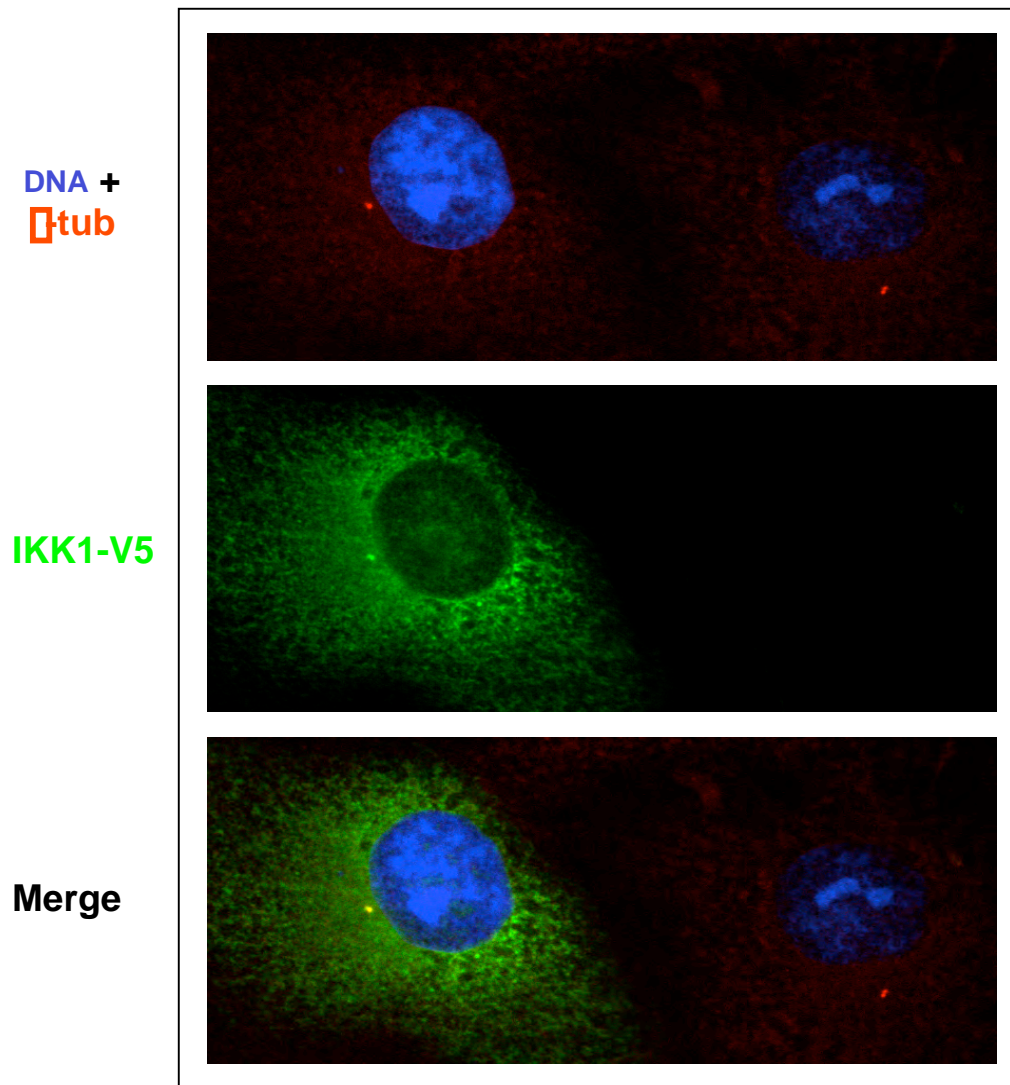
V5-epitope-tagged IKK localizes to the centrosome. V5-epitope-tagged bIKK1 (IKK1-V5) was overexpressed in COS-7 by transient transfection. Anti-γ-tubulin antibodies were used to reveal the centrosome (red). Anti-V5 antibodies were used to detect V5-tagged recombinant IKK1 (green), which is only expressed in the cell on the left. "Merge" represents an overlay of the two images.

Supplemental Figure 3
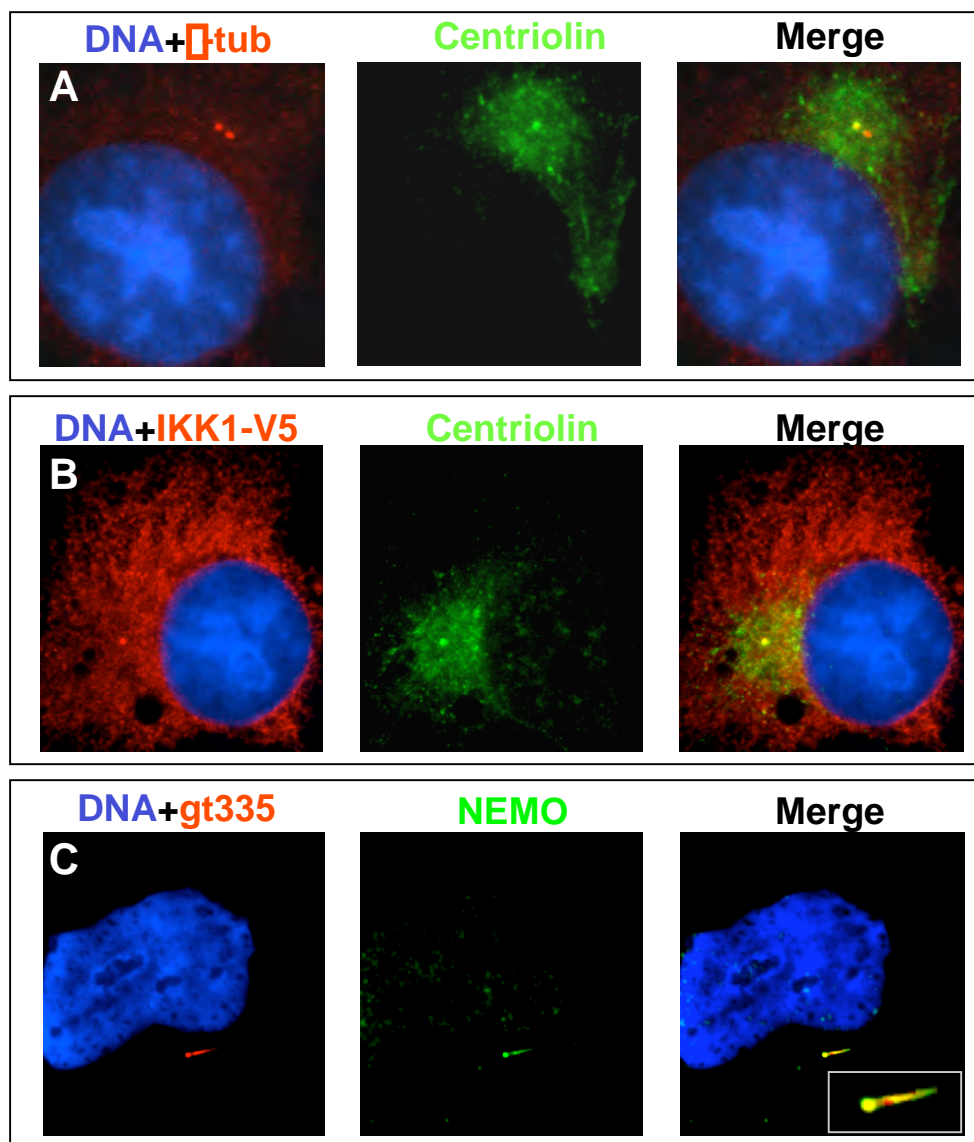
IKK colocalizes with the maternal centriole and the primary cilium.
(A) Immunofluorescence microscopy showing the association of centriolin, a protein of the maternal centriole (*S2*) (green), with one of two centrosomes (detected by anti-γ-tubulin, red). Nuclear DNA (blue) was stained with Hoechst DNA dye. "Merge" represents an overlay of the two images
(B) In transiently transfected COS-7 cells, V5-epitope-tagged IKK1 colocalizes with centriolin (green).
(C) NEMO (green) can be found in association with the maternal centriole and the primary cilium (detected by an antibody against polyglutamylated tubulin, gt335, red), which grows from the maternal centriole in serum-starved hTERT-RPE-1 cells. The inset shows a higher magnification of the primary cilium.

Supplemental Figure 4

A

siLamin   siNEMO   siCentrin-2

B

Phase contrast   DNA
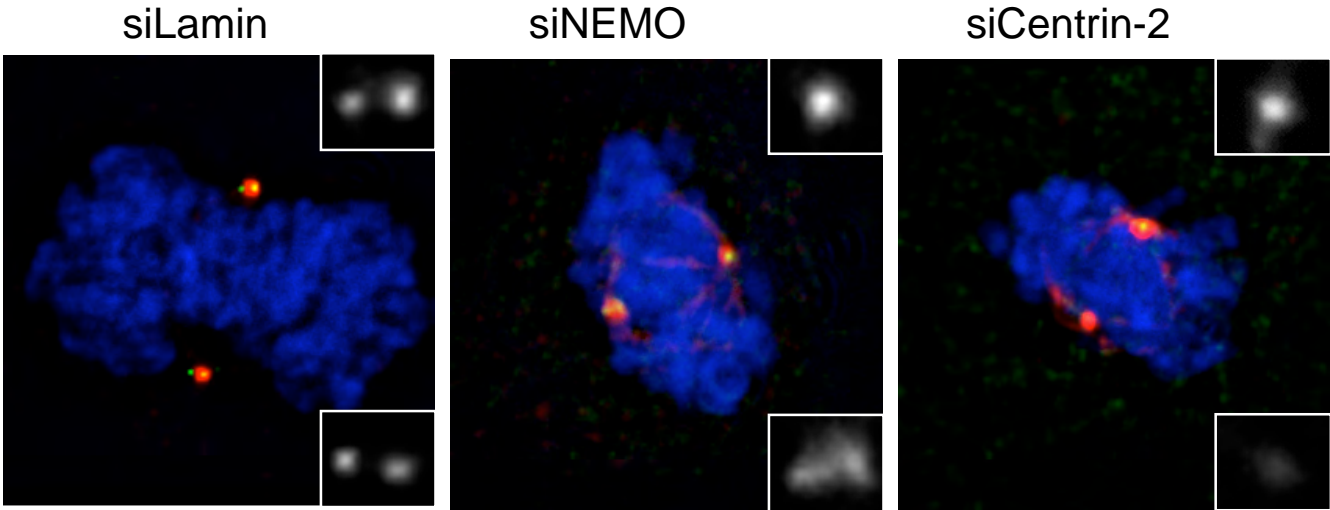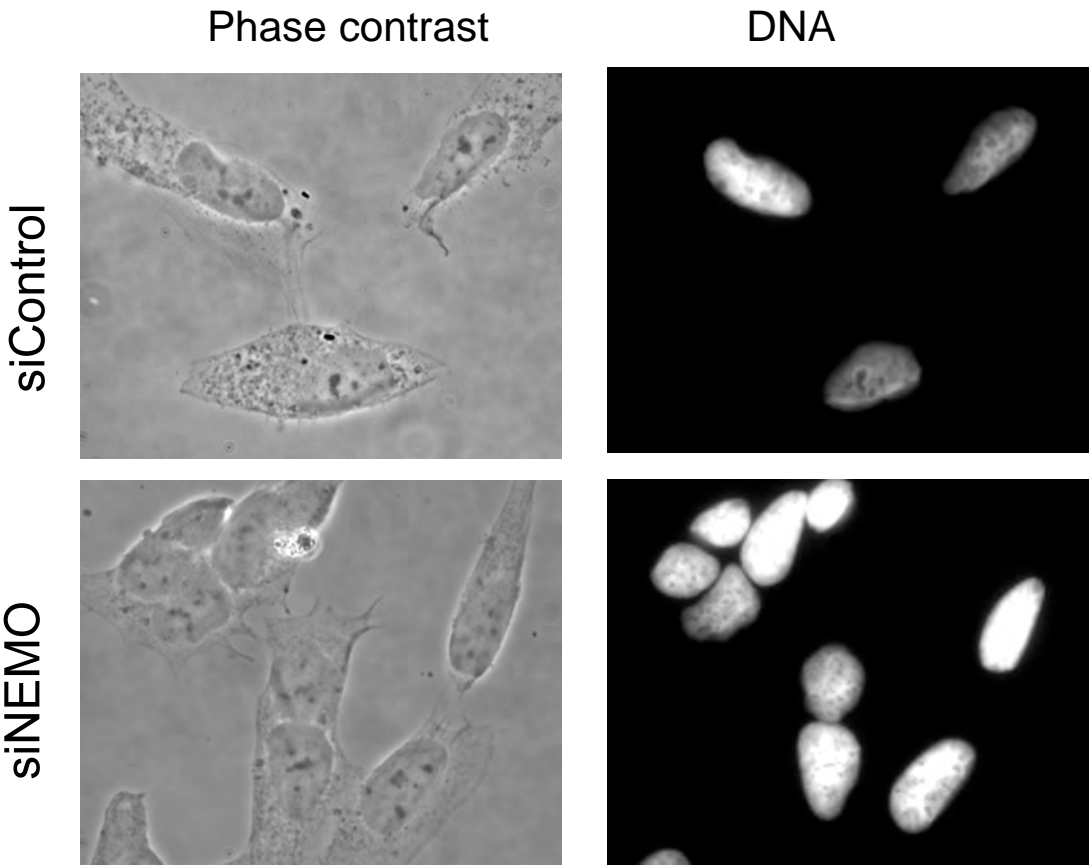
siControl

siNEMO

Treatment of HeLa cells with siNEMO inhibits cenrtriole duplication and causes defects in cytokinesis.

(A) In control HeLa cells treated for 48 h with siLamin, antibodies directed against centrin-2 (green) detect a pair of centrioles at each mitotic spindle pole (stained with anti-γ-tubulin, red); middle panel: siNEMO-treated cells exhibit centriolar defects; right panel: in cells treated with siCentrin-2 (included as a positive control) spindle poles lacking centrioles can also be found. The insets show the centrin-2-stained centrioles for each spindle pole.

B: HeLa cells treated for 48 h with control siRNA (siControl) possessing a single nucleus per cell (top panels); siNEMO-treated cells are often multinucleate (lower panel). Phase contrast and DNA staining are shown as indicated.

Materials and Methods

Cell culture and transfection with bIKK1-V5. COS-7, HeLa, U2OS, hTERT-RPE-1(CLONTECH Laboratories) and Jurkat T-cells were maintained in culture according to the recommendations issued by the American Type Culture Collection for each cell line. A cDNA fragment encoding full-length bovine IKK1 *(S1)* was subcloned into pcDNA 3.1/V5-HIS (Invitrogen) using the *BamHI* and *NotI* restriction enzyme sites. This construct was transfected into COS-7 cells using the Gene Jammer transfection reagent (Stratagene) according to the manufacturer's recommendations.

Digital immunofluorescence microscopy. Antibody reagents: rabbit anti-IKK1 (Santa Cruz, sc 7218, 1/50), rabbit anti-IKK2 (Santa Cruz, sc 7607, 1/50), rabbit anti-NEMO (Santa Cruz sc 8330, 1/50), mouse anti-α- and γ-tubulin (Sigma-Aldrich, 1/500), mouse anti-AIM-1 (BD Biosciences, 1/200), rabbit anti-V5 (MBL, 1/200), mouse anti-V5 (Invitrogen, 1/500), rabbit anti-centriolin *(S2)*, rabbit anti-P-IKK (Cell Signaling, 1/50), rabbit anti-P-IκBα (Cell Signaling, 1/50), goat anti-rabbit Texas Red (Vector laboratories, 1/500), goat anti-rabbit Alexa Fluor 488 (Molecular Probes, 1/1500), goat anti-mouse Alexa Fluor 488 (Molecular Probes, 1/1500) and goat anti-mouse Texas Red (Vector laboratories, 1/800). Immunofluorescence (IF) staining: 2×10^4 cells were seeded onto glass coverslips (12mm diameter). After O/N culture, coverslips were rinsed in PBS and cells were fixed in 100% methanol at -20°C for 5min and then processed for IF as described previously *(S3)*. Slides were analyzed using a Nikon Eclipse microscope and images generated by digital imaging using Openlab 3.1.1. software (Improvision) and Metamorph (Universal Imaging Corp.). Quantitation of centrosomal protein levels for Fig. 2A was performed as described *(S4)*. For Fig. 3A and 3F, the level of P-IKK in captured two-dimensional digital images was quantified by measuring the mean signal intensity per pixel for the area covering individual centrosomes, using Openlab 3.1.1 software; background values were recorded by the same approach in another area of the cytoplasm and subtracted.

siRNA. siRNAs targeting NEMO, Lamin A/C, Centrin-2 and Ninein mRNAs were generated as complementary single-stranded 19-mer siRNAs with 3' dTdT overhangs (Dharmacon Research), deprotected, annealed, and delivered into cells from a 400mM stock using Oligofectamine (Invitrogen). The nucleotides targeted were as follows (according to the coding sequence): 608-630 (Lamin A/C, accession No.: NM_005572, *S5*), 385-405 (NEMO, accession No.: NM_003639) and 80-100 (Centrin-2, accession No.: NM_004344, *S6*). siControl consisted of nonsense oligonucleotides devoid of inhibitory activity.

Immunoblotting. Standard methods were applied for immunoblot analysis *(S5)*. Antibody reagents: rabbit anti-NEMO (Santa Cruz sc 8330, 1/500), sheep anti-α,β-tubulin (Cytoskeleton, 1:1000), anti-rabbit-HRP (Dako, 1/2000), anti-sheep-HRP (Cytoskeleton, 1/2000).

Centrosome/centriole duplication assays. 24h after siRNA delivery, U2-OS cells were treated with 4mM hydroxyurea and IF analysis was performed using primary antibodies against α- and γ-tubulin (Sigma-Aldrich). To investigate centriole duplication, HeLa cells were treated with siRNA and analyzed by IF as described *(S6)*.

Inhibitor experiments. Exponentially growing COS-7 cells (2×10^6 in a 150cm^2 tissue culture flask) were treated with 0.1μg/ml nocodazole (Sigma-Aldrich) for 16h. Cells collected by mitotic shake-off were washed 3x in PBS and released for 2h in the presence or absence of 100μM prostaglandin A1 (PGA1, Cayman Chemical Co.), 30μM 15-deoxy-Δ12,14-prostaglandin J2 (PGJ2, Cayman Chemical Co.), 2mM sulfasalazine (Sigma-Aldrich) or 1μg/ml actinomycin D (Sigma-Aldrich) for 2h. Harvested cells were washed in ice-cold PBS, fixed in –20°C 70% ethanol for at least 2h and washed again in PBS. The pellet was resuspended in 100μl PBS containing 200μg/ml RNAse A. After incubation at RT for 30min, 400μl of propidium iodide solution (0.1% NP-40, 0.2 mg/ml RNAse A, 3.4 mM Tris pH 7.5, 30 mM propidium iodide) were added. Flow-cytometric analysis was carried out using FACScan (BD Biosciences). To test the effects of the cdk inhibitor

butyrolactone I, COS-7 cells were first arrested in early S-phase by a double-thymidine block (18 h culture in 3.5 mM thymidine; 9 h in the absence, and another 18 h in 3.5 mM thymidine). The cells were released by washing out the thymidine and cultured for 8h followed by 1h in the presence or absence of 50μM butyrolactone I (Sigma-Aldrich).

Supplemental references

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S2.   A. Gromley *et al.*, *J Cell Biol*. **161**, 535 (2003).

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S4.   J. B. Dictenberg *et al.*, *J Cell Biol*. **141**, 163 (1998).

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S6.   J. L. Salisbury *et al.*, *Curr Biol*. **12**, 1287 (2002).

Chromatin remodeling proteins interact with pericentrin to regulate centrosome integrity

James Sillibourne*, Manisha Sinah and Stephen Doxsey

Program in Molecular Medicine, University of Massachusetts, 373 Plantation Street, Worcester, MA 01605, USA.

*Current Address: Institut Curie, Section Recherche UMR 144 du CNRS, 26 rue d'Ulm, Paris 75248, France

ABSTRACT

Pericentrin is an integral centrosomal component that anchors regulatory and structural molecules to centrosomes. In a yeast two-hybrid screen with pericentrin we identified chromodomain helicase DNA-binding protein 4 (CHD4/Mi2β). CHD4 is part of the multiprotein nucleosome remodelling deacetylase (NuRD) complex. We show that many NuRD components interacted with pericentrin by co-immunoprecipitation and that they localized to centrosomes and midbodies. Over-expression of the pericentrin-binding domain of CHD4 or another family member (CHD3) dissociated pericentrin from centrosomes. Depletion of CHD3, but not CHD4, by RNA interference dissociated pericentrin, γ-tubulin and other centrosome components from centrosomes. Microtubule nucleation/organization, cell morphology and nuclear centration were disrupted in CHD3-depleted cells. Spindles were disorganized, the majority showing a prometaphase-like configuration. Time-lapse imaging revealed mitotic failure prior to chromosome segregation and cytokinesis failure. We conclude that pericentrin forms a complex(es) with CHD3 and CHD4, but a distinct CHD3-pericentrin complex is required for centrosomal anchoring of pericentrin/γ-tubulin and for centrosome integrity.

INTRODUCTION

Centrosomes are the major microtubule organizing centers (MTOC) in animal cells and play a pivotal role in cell cycle progression, bipolar spindle formation and cytokinesis (Doxsey *et al.*, 2005). A centrosome consists of a pair of centrioles surrounded by a protein matrix or pericentriolar material (PCM). Pericentrin localizes to the PCM and is responsible for anchoring both regulatory and structural proteins at the centrosome. A number of pericentrin-interacting proteins have been identified including GCP2 and 3 of the γ-tubulin ring complex (γ-TuRC) (Dictenberg *et al.*, 1998; Zimmerman *et al.*, 2004) the light intermediate chain of cytoplasmic dynein (Purohit *et al.*, 1999), the kinases PKA and PKCβII (Diviani *et al.*, 2000; Chen *et al.*, 2004; Zimmerman *et al.*, 2004) and PCM-1 (Li *et al.*, 2001). Pericentrin and its homologs in yeast, Drosophila and Aspergillus has been shown to be involved in microtubule and spindle organization and function in some systems (Kilmartin and Goh, 1996; Purohit *et al.*, 1999; Flory *et al.*, 2002; Kawaguchi and Zheng, 2004; Zimmerman *et al.*, 2004) but not others (Martinez-Campos *et al.*, 2004). Pericentrin also appears to play a role in the assembly/maintenance of primary cilia and flagella (Jurczyk *et al.*, 2004; Martinez-Campos *et al.*, 2004).

Chromodomain helicase DNA-binding (CHD) proteins are related by the presence of two chromatin organization modifier domains (chromodomains), a SWI/SNF-type ATPase domain and DNA-binding motif. Chromodomains (30-50 amino acids) mediate protein/DNA or protein/protein interactions (Brehm *et al.*, 2004). The human CHD family contains at least nine members that can be grouped into subfamilies based on

sequence homology: CHD1 and CHD2; CHD3, CHD4 and CHD5; and CHD6-CHD9 (Woodage *et al.*, 1997; Aubry *et al.*, 1998; Schuster and Stoger, 2002; Thompson *et al.*, 2003; Flaus *et al.*, 2006). Homologs of CHD proteins have been found in *S. cerevisiae*, *S. pombe*, *D. melanogaster*, *C. elegans* and *M. musculus* (von Zelewsky *et al.*, 2000; Jae Yoo *et al.*, 2002) . A single CHD gene, CHD1, is present in *S.cerevesiae*, while *S. pombe* contains two, Hrp1 and Hrp3 (Jin *et al.*, 1998; Tran *et al.*, 2000; Yoo *et al.*, 2000; Jae Yoo *et al.*, 2002). *C. elegans* and *D. melanogaster* both possess at least two different CHD genes (Woodage *et al.*, 1997).

CHD4, and or CHD3, have been found in multiple complexes including the nucleosome remodeling deacetylase (NuRD) complex, a second complex regulating the deacetylation and inactivation of p53, a third involved in loading cohesin onto chromatin (Wade *et al.*, 1998; Zhang *et al.*, 1998; Luo *et al.*, 2000; Wang and Zhang, 2001; Hakimi *et al.*, 2002) and a forth potentially involved in the DNA damage response and silencing genes involved regulating cell cycle progression (Schmidt and Schreiber, 1999).

In addition to CHD4 (and CHD3), the NuRD complex contains: histone deacetylases 1 and 2, retinoblastinoma associated proteins 46 and 48 (RbAp46/48), methyl-CpG-binding domain-containing protein 3 (MBD3) and a metastasis associated protein (MTA) subunit (Tong *et al.*, 1998; Wade *et al.*, 1998; Zhang *et al.*, 1998; Bowen *et al.*, 2004). CHD4 hydrolyzes ATP in a DNA-dependent manner in vitro and this activity is greatly increased when DNA is wrapped around histone octamers (nucleosomes) (Wang and Zhang, 2001). This activity is thought to be important for the complex to act as a

transcriptional repressor, perhaps by utilizing energy of ATP hydrolysis to remodel nucleosomes. This could allow HDACs to access and deacetylate histone acetyl-lysine residues thus compacting nucleosomes and initiating transcriptional repression. A NuRD complex containing MTA3 was identified that regulates E-cadherin expression by controlling the expression of the negative regulator Snail (Fujita *et al.*, 2004), thus identifying the first gene regulated by the NuRD complex. It is unclear if the NuRD is a global repressor or a repressor of a subset of genes. The NuRD complex characterized by Fujita et al, which contained MTA3 exclusively, suggests that MTA subunits might confer targeting specificity to the complex and that the activity of the NuRD might be restricted to the repression of specific genes.

In this paper we identify an interaction between pericentrin and CHD3/4 and other NuRD components, and suggest that these proteins form complexes distinct from the NuRD. We show that CHD3, CHD4, MTA2 are components of the centrosome and that functional abrogation of CHD3 and to some extent CHD4, disrupts centrosome integrity and function, microtubule organization and mitotic progression.

RESULTS

Identification of CHD3 and CHD4 as pericentrin-interacting proteins

To identify pericentrin-interacting proteins a region of mouse pericentrin A corresponding to residues 1340-1756 (Zimmerman *et al.*, 2004) was used as bait in the

yeast two-hybrid system to screen a human testis cDNA expression library. Among the pericentrin-interacting clones identified was the C-terminal domain of CHD4 (residues 1577-1912), a member of a protein family that localizes to the nucleus, functions in transcriptional regulation and contains two chromodomains and a SWI/SNF helicase domain (Woodage *et al.*, 1997). CHD3, a closely related member of the chromodomain-SWI/SNF helicase family, also interacted with pericentrin in the two-hybrid system (residues 1566-1966, Fig. 1A). Deletion mapping (Fig. S1) narrowed the pericentrin interacting domain of CHD3 to amino acid numbers 1687-1880; this region shared 81% sequence identity and 92% similarity with CHD4.

Immunoprecipitation experiments confirmed the two-hybrid interactions. In COS-7 cells transiently expressing HA-tagged-pericentrin and FLAG-tagged-CHD4, immunoprecipitation of FLAG-CHD4 co-precipitated HA-pericentrin (Fig. 1B); the upper band of the CHD4 doublet could represent a post-translationally modified form of the protein, potentially sumoylation, as this has been recently demonstrated to occur *in vitro* (Gocke *et al.*, 2005). We then showed that overexpressed FLAG-CHD4 co-precipitated endogenous pericentrin (Fig. 1C) and conversely, that overexpressed FLAG-tagged pericentrin co-precipitated endogenous forms of both CHD3 and CHD4 (Fig. 1D). Finally, immunoprecipitation of endogenous CHD3 and CHD4 from HeLa cell extracts co-precipitated endogenous pericentrin (Fig. 1E).

Other NuRD components were tested for their ability to interact with pericentrin. FLAG-tagged pericentrin expressed in COS cell co-precipitated a significant amount of the

soluble MTA2 and less of the soluble RpAp46 and MBD3. It is possible that RpAp46 and MBD3 interact with pericentrin with a lower affinity or are transiently associated with the pericentrin-CHD3/4 complex.

NuRD components localize to the centrosome

It has been shown that a number of NuRD complex components localize to the centrosome/spindle pole during mitosis including CHD3/4 (Mi2), MBD3 and HDAC1 (Chadwick and Willard, 2002; Sakai *et al.*, 2002). We re-examined these results by investigating localization of NuRD components throughout the cell cycle in RPE-1 cells using antibodies specific for CHD3, CHD4, MTA2 and RpAp46. The predominant location of all proteins in interphase was the nucleus and to a lesser extent, the cytoplasm, but we also detected distinct staining of CHD3, CHD4 and MTA2 at centrosomes (Fig. 2). This staining was enhanced when the cytoplasm was extracted with detergent before fixation (data not shown). During prophase the staining intensity of CHD3, CHD4 and MTA2 diminished in the nucleus. Centrosome staining of CHD3 and MTA2 remained throughout mitosis, whereas CHD4 appeared to dissociate from the centrosome; CHD3/4 and MTA2 also appeared at the midbody during cytokinesis. RpAp46 did not localize to the centrosome at any cell cycle stage but was enriched on the spindle during metaphase (Fig. 2).

To determine whether NuRD components were core components of centrosomes or if they simply accumulated at the minus ends of microtubules, we examined their localization in the presence of the microtubule depolymerizing drug nocodazole. In the

absence of centrosomal microtubules CHD3, CHD4 and MTA2 retained their association with the centrosome in interphase and mitosis demonstrating a stable interaction with this organelle (data not shown).

Expression of the C-termini of CHD3 and CHD4 displaces centrosomal pericentrin

Pericentrin acts a scaffold for the γ-TuSC and kinases such as PKA and PKCβII at the centrosome (Dictenberg *et al.*, 1998; Young *et al.*, 2000; Takahashi *et al.*, 2002; Chen *et al.*, 2004; Zimmerman *et al.*, 2004). To test whether pericentrin served as a centrosomal scaffold for CHD3/4 and other NuRD components, we attempted to disrupt the pericentrin-CHD3/4 interaction by expressing the pericentrin-interacting C-terminal domains of CHD3 and CHD4 (CT-CHD3 and –CHD4) in HeLa cells. We unexpectedly found that pericentrin (not CHD3/4) was mislocalized from centrosomes under these conditions (Fig. 3). A significant percentage (~40%) of CT-CHD3 or CT-CHD4 expressing cells contained no detectable centrosomal pericentrin (Fig. 3B) and other cells stained weakly or showed a tiny focus of pericentrin staining (24.5-29.5%). In contrast, control cells expressing GFP always showed robust pericentrin staining that appeared as one or two foci. The dramatic loss of centrosomal pericentrin occurred rapidly (within 6 hours) following electroporation of cDNA encoding CT-CHD3, indicating that transcriptional activity of the NuRD complex may not play a major role in the centrosome disruption phenotype. In some cells, overexpressed CT-CHD3 and CT-CHD4 localized to the centrosome and these centrosomes showed a concomitant reduction in centrosomal pericentrin. This suggested that centrosomal recruitment of over-expressed CT-CHD3 and –CHD4 displaced or prevented the localization of pericentrin at this site.

Taken together these results indicate that the C-termini of CHD3 and CHD4 contain a functional pericentrin-binding domain.

siRNA-mediated depletion of CHD3 mislocalizes centrosome proteins

We next examined the effect of depleting CHD3 and CHD4 on centrosome integrity. siRNA targeting CHD3 or CHD4 showed depletion of the target protein by immunoblotting; other NuRD components were not affected (Fig. 4A). Immunofluorescence analysis showed that target proteins were lost from nuclei and centrosomes in interphase cells (Fig. 4B).

Cells depleted of CHD3 showed reduced centrosomal staining with 5051, a patient autoimmune serum that recognizes several centrosome proteins including pericentrin (Doxsey *et al.*, 1994) and centriolin (Gromley *et al.*, 2003), indicating that these proteins were lost from the centrosome (Fig. 4B). In fact, pericentrin immunofluorescence images and quantitative analysis showed that pericentrin was lost from centrosomes in cells depleted of CHD3 (Fig. 5A, left). In contrast, no reduction in centrosomal pericentrin levels were observed in cells treated with siRNAs targeting CHD4 or lamins (Fig. 5A, middle and right). Other experiments demonstrated that centrosome levels of γ-tubulin, a centrosome protein that binds pericentrin through its association with the γ-tubulin ring complex (Zimmerman *et al.*, 2004), was reduced by a moderate amount upon treatment with siRNAs against CHD3 but not CHD4 or lamins (Fig. 5B). This suggested that disruption of centrosomal pericentrin displaced a fraction of γ-tubulin from interphase centrosomes (Zimmerman *et al.*, 2004) and that other proteins likely anchor the

remaining fraction of γ-tubulin to interphase centrosomes (Takahashi *et al.*, 2002). Centrin-1 staining in CHD3-depleted cells was not different from controls, indicating that the core structure of the centrosome (centrioles) was still intact (Fig. 5C). These results combined with those from transient transfection CHD3 and CHD4 C-termini suggest that both proteins are able to interact with pericentrin, but only CHD3 is responsible for anchoring centrosomal components. RNAi data indicated that CHD4 is not involved in this function, but overexpression of the pericentrin-binding domain of this protein, which shares 92% homology to that of CHD3, produces a similar phenotype as CT-CHD3 overexpression because it presumably interferes with function of endogenous CHD3.

Microtubule organization and regrowth is altered in CHD3 siRNA-treated cells

CHD3/4-dependent mislocalization of centrosomal pericentrin and γ-tubulin, which both play a role in microtubule nucleation and organization (Zimmerman *et al.*, 2004), suggested a role for the NuRD complex in these processes. We first examined microtubule organization in cells expressing the C-termini of CHD3 and CHD4 following nocodazole treatment and release. Six hours after electroporation of cDNAs encoding the C-termini of CHD3 or CHD4 microtubule organization was dramatically perturbed in 84% of RPE cells, whereas only 3% of GFP-transfected cells showed any change in microtubule organization (Fig. 6A). Cells showed a dramatic reduction in the total number of microtubules, which usually formed a random meshwork rather than a radial array as seen in GFP-expressing controls. Moreover, the cells were not as flat and spread-out as controls presumably due to disruption of the microtubule cytoskeleton. These data suggested that over-expression of the CHD3 or CHD4 C-termini disrupted microtubule

arrays by displacing centrosomal components and consequently compromising the ability of centrosomes to organize a microtubule array.

Profound defects in microtubule organization were also observed in microtubule regrow assays when CHD3 was depleted (Fig. 6B). About 70% of CHD3 siRNA-treated cells showed phenotypes consistent with defects in microtubule nucleation, anchoring and general organization. Because protein depletion occurs in only 75-80% of the cell population, the penetrance of the microtubule disruption phenotype is near complete. CHD3-depleted cells had microtubules that were fewer in number, not properly organized into radial arrays and absent from large areas of the cytoplasm. In addition, a slight delay in nucleation was observed, as cells fixed five minutes after nocodazole washout still contained unpolymerized tubulin. Microtubules were sometimes organized into asters independent of centrosomes, which might have either formed spontaneously within the cytoplasm or been organized by centrosomes then released (Fig. 6B). Centrosome-nucleated asters had many fewer microtubules, many of which seemed to have lost their centrosome attachment (Fig. 6B, 5 minutes). When present, centrosome-anchored microtubules were as long as controls suggesting that their polymerization rate was unaltered (Fig. 6B). Further images of regrowing microtubules in CHD3 siRNA-treated cells are shown in figure S3. Microtubules were often found at the cell periphery unattached to centrosomes, suggesting that they had been nucleated ectopically at these sites or were centrosome nucleated, released and transported to these sites (Fig S3). Nuclei were often displaced from a central position presumably from loss of microtubule organization. Ten minutes after nocodazole washout microtubules appeared to be

dramatically curved at the cell periphery and often formed bundles around the nucleus (Fig. 6B).

Some CHD4-depleted cells exhibited poorly formed microtubule arrays (24%), a significantly higher percentage than lamin siRNA-treated cells (1-2%), but neither ectopic microtubule nucleation nor a delay in nucleation was observed after five minutes of regrowth.

CHD3 depletion causes mitotic- and cytokinesis-failure

We next investigated the effect of CHD3 depletion on mitotic spindle integrity and function. Reduction of CHD3 levels induced spindle defects in 59.6% of mitotic cells (Fig. 7, Fig. S2). The most prominent phenotype was a disrupted prometaphase-like configuration with a dramatic reduction in spindle microtubules and misalignment of chromosomes within spindles (Fig. 7A). We also observed a significant number of half-, monopolar- and tripolar-spindles as well as spindles with lagging chromosomes. In contrast, these types of spindle defects comprised only 3.6% of the control HeLa cell population (lamin siRNA-treated) and 14.4% in CHD4 depleted cells (Fig. 7B). The percentage of spindle defects in CHD4 depleted cells was significantly higher than controls but less than CHD3 depleted cells, and did not include the predominant prometaphase-like defects seen after CHD3 depletion.

Consistent with the apparent loss of microtubule polymer in spindles of CHD3 depleted cells was a reduction in the amount of γ-tubulin at the spindle poles and on the spindle,

and a concomitant increase in the cytoplasm (Fig. 7C). There appeared to be a selective loss of γ-tubulin from the poles as the total cellular level of the protein, assessed by immunoblotting, was similar to controls (data not shown). Spindle pole levels of γ-tubulin in CHD4 depleted cells were similar to controls, suggesting that the modest increase in microtubule disorganization and spindle defects seen in these cells occurred by a mechanism distinct from the CHD3 induced defects.

To further investigate the fate of CHD3-depleted cells during mitosis, we performed time-lapse imaging using phase contrast microscopy. Cells were continuously examined for 23-28 hours (Fig. 8, movies S1 and S2). Control cells (lamin A/C depleted) completed the transition from cell rounding to anaphase in 37.8 minutes and 27/28 completed mitosis normally (Fig. 8A, D); one formed a tripolar spindle and gave rise to three progeny. In contrast, CHD3 depleted cells that completed cell division took an average of 265 minutes to transit the same time period. Many CHD3 depleted cells failed to segregate their chromatids, exited mitosis and reattached to the substrate as mononuclear polyploid cells (Fig. 8C). Others attempted to divide but failed in cytokinesis to form binucleated cells (Fig. 8B). While all control cells entered mitosis during the time that cells were examined, only half of the CHD3 depleted cells entered mitosis in the same time frame. This suggested a delay at a cell cycle stage prior to mitosis. It has been shown that cells failing in cytokinesis arrest in G1 (Hinchcliffe *et al.*, 2001). However some CHD3-depleted cells that failed cytokinesis entered a second round of division indicating that cell cycle delay/arrest was at another cell cycle stage.

DISCUSSION

Identification of novel pericentrin-interacting proteins

We have shown that pericentrin interacts with members of the NuRD complex and that the NuRD components CHD3, CHD4 and MTA2 localize to centrosomes during interphase. Differences in the localization patterns of NuRD components during mitosis were observed, CHD3 and MTA2 remained associated with the centrosome, whereas CHD4 dissociated and was absent from metaphase until telophase. Other NuRD components have been shown to localize to spindle poles (MBD3, HDAC1) (Chadwick and Willard, 2002; Sakai *et al.*, 2002), suggesting the entire complex may be located at this site, in addition to its nuclear localization.

Pericentrin-NuRD complexes

Our results suggest that pericentrin interacts with a subset of NuRD components indicating that this complex may be different from other NuRD complexes. There is some controversy within the chromatin field as to whether CHD3 is present within the NuRD complex. Zhang et al (1998) isolated NuRD complexes from HeLa nuclear extracts and identified 43 peptides by mass spectrometric analysis, the majority of which corresponded to sequences conserved between CHD3 and CHD4, although 4 peptides specifically derived from CHD4 (Mi2β), but none from CHD3, were identified (Zhang *et al.*, 1998). Other groups found that CHD3 was present in the NuRD, but was less abundant than CHD4, suggesting that CHD3 either formed distinct complexes or was a minor component of the NuRD. Taken together these data suggest that it is possible that

pericentrin specifically forms a complex with CHD3 that is distinct from the NuRD. Differences in the abundances of NuRD components within the pericentrin complex support this argument, as extremely low amounts of MBD3 and RpAp46 were found to be present, suggesting that these were not core components and interacted weakly. Our data also indicates that CHD3 and CHD4 probably form different complexes with pericentrin, which have alternative functions. CHD3 appears to play a role in regulating protein anchorage at the centrosome both in interphase and mitotic cells, whereas CHD4 has no such role, not at least in the presence of CHD3.

Does CHD3 directly anchor pericentrin at centrosomes?

Our transient transfection experiments support a direct role for CHD3/NuRD in anchoring pericentrin to the centrosome. The over-expression of the pericentrin-binding domains of either CHD3 or CHD4 displaced endogenous pericentrin from the centrosome within 6 hours, a time that is likely too short for significant gene expression. Furthermore, over-expressed proteins were distributed throughout the cytoplasm and presumably did not disrupt the function of the NuRD complex within the nucleus, as they were excluded from this cellular compartment. However, an alternative model whereby anchoring occurs indirectly via another protein transcriptionally regulated by the NuRD can not be excluded.

A link between the centrosome and nucleus?

The interaction between pericentrin, a centrosomal protein, and CHD3/4 and MTA2, components of a nuclear complex, is not without precedence. This interaction joins a

growing list of liaisons between nuclear and centrosomal proteins, for example: centrin-2 is part of the XPC, TACC (transforming acidic coiled-coil) proteins 1-3 directly bind to the histone acetyltransferase hGCN5L2, the drosophila protein CP190 forms part of the gypsy chromatin insulator, hEg5 is within a N-CoR repressor complex and (Araki *et al.*, 2001; Yoon *et al.*, 2003; Gangisetty *et al.*, 2004; Pai *et al.*, 2004; Doxsey *et al.*, 2005). These complexes have a diverse range of functions. XPC is involved in nucleotide excision repair (NER) and is responsible for detecting damaged DNA in global genome nucleotide excision repair (GGR). The role of hEg5 within the N-CoR repressor complex, if it is a genuine component, is unknown. CP190 was found to be an essential component of the gypsy chromatin insulator and localizes to centrosomes, although mutation of this gene appeared to have no affect upon centrosome function (Pai *et al.*, 2004). Human TACC proteins localize to the nucleus and centrosome, although TACC1 and 3 associate with the centrosome only during mitosis (Gergely *et al.*, 2000). On one hand, TACC proteins are involved in microtubule organization and enhance recruitment of microtubules to the mitotic spindle (Gergely *et al.*, 2000). On the other hand, they appear to regulate gene expression via their interaction with the HAT hGCN5L2, which associates with chromatin remodeling components (Gangisetty *et al.*, 2004). Abnormal expression of TACC proteins contributes to the development of multiple myeloma, breast and gastric cancer, an effect that could occur via two different mechanisms, aberrant gene expression or spindle dysfunction (Gergely *et al.*, 2000; Conte *et al.*, 2002; Conte *et al.*, 2003; Gangisetty *et al.*, 2004). Thus, there are clear parallels between TACC proteins and CHD3/4, as both have similar localization patterns, play roles in transcription regulation

and alter microtubule patterns. Together, these results indicate that nuclear proteins play a role in regulating the activity of the centrosome.

Does pericentrin regulate gene expression?

RNAi experiments demonstrated that the nuclear protein CHD3 anchors pericentrin to the centrosome and affect microtubule and spindle function, which provokes the question: does the centrosome protein pericentrin function in the nucleus? A recent study demonstrated that cells treated with leptomycin B, an inhibitor of nuclear export, induced accumulation of pericentrin within the nucleus (Keryer *et al.*, 2003). This indicates that pericentrin is able to traverse the nuclear envelope and may have a function within the nucleus but is rapidly shuttled out of this compartment.

Other evidence suggests a role for pericentrin in the nucleus. A NuRD complex, containing MTA3, has recently been found to play a role in governing the expression of the E-cadherin by controlling the level of Snail a transcriptional repressor of this gene. It is interesting to note, that a cDNA encoding a protein sharing homology with the transcriptional repressor kaiso was identified as a pericentrin-interacting protein in the yeast two-hybrid screen. If this kaiso-like protein is a bona fide interacting protein, it would further support the idea that pericentrin plays a role in regulating gene expression by associating with specific transcription factors and components of the NuRD complex.

Moreover, a recent paper demonstrating that pericentrin colocalizes with over-expressed SUMO in small nuclear dots suggests that pericentrin interacts with or is modified by the

small ubiquitin modifier (SUMO) (Cheng *et al.*, 2005). In addition, there is evidence providing a link between SUMO and CHD3. A yeast two-hybrid screen for p73-interacting proteins identified SUMO and CHD3 as potential partners; subsequent cross tests demonstrated that the C-terminus of CHD3 interacted with SUMO (Minty *et al.*, 2000). CHD3 and CHD4 have been recently identified as a target of SUMO modification *in vitro* indicating that these proteins are potentially modified *in vivo* (Gocke *et al.*, 2005).

In this paper we have presented evidence demonstrating that CHD3/4 are centrosomal proteins and that ablation of CHD3 causes the subsequent loss of pericentrin from the centrosome. This indicates that CHD3 is involved in regulating the level of pericentrin and other components, by a yet undefined mechanism, at the centrosome. Several questions remain unanswered, including how is CHD3 anchored to the centrosome, what is the function of CHD4 at the centrosome and how does CHD3 control localization of centrosomal components? A number of models for the mechanism by which CHD3 controls pericentrin abundance (and other centrosomal components) at the centrosome can be put forward: CHD3, acting as part of the NuRD complex, could directly repress pericentrin gene expression down-regulating the amount of protein within the cell; alternatively, CHD3 could co-ordinate the activity of proteins involved in post-translation modification such acetylation/deacetylation or SUMOylation. The first model can not be excluded, but RNAi silencing of CHD3 gene results no overall change in pericentrin levels suggesting that CHD3 is not involved in regulating pericentrin gene expression. It is tempting to speculate that CHD3 coordinates centrosome assembly by post-

translational modification such as SUMOylation, as this has been shown to prevent protein ubiquitination and subsequent degradation and alter protein activity and intracellular localization.

METHODS

Yeast Two-Hybrid Screen

The yeast strain AH109 was transformed with a GAL4 DNA-binding domain fusion vector, pGBKT7 (Clontech), containing residues 1340-1756 of murine pericentrin. After determining that the GAL4 DBD/pericentrin fusion failed to auto-transactivate the reporter genes, a 50 ml culture was grown overnight and then mated with the yeast strain Y187, which had been pre-transformed with a human testes cDNA library (Clontech). Diploid clones were plated out onto to synthetic defined medium lacking leucine, tryptophan, histidine and adenine to select for positive interactants.

cDNA clones, cloning techniques and expression constructs

Complementary DNAs (cDNAs) encoding CHD3, CHD4 and pericentrin were obtained from the following sources: CHD3 C-terminal sequence from IMAGE clone 642405 (IMAGE consortium); CHD4 human testis cDNA library described above; pericentrin as described before. Sequences encoding pericentrin, CHD3 and CHD4 were amplified by PCR using Pfu Turbo DNA polymerase (Stratagene), cloned into a donor vector of the Creator system (Clontech) and sequenced to verify the fidelity of the amplifying enzyme (Applied Biosystems). Coding sequences were transferred, by Cre-mediated recombination, into a range of expression vectors that included pLP-GBK-T7 (Clontech), pLP-CMV-myc (Clontech) and an existing FLAG-tagged vector that was converted for use with the system. The recombination protocol was as follows: 200 ng of each vector (donor and acceptor) were incubated at 37°C for 1 hour in 1X Cre recombinase buffer with 1 unit of Cre recombinase (NEB), the enzyme was then heat inactivated at 70°C for

5 min and the reaction allowed to slowly cool to room temperature. Chemically competent DH5α were transformed with 2 µl of heat-inactivated recombination reaction.

Cell Culture and transfection

COS and HeLa cells were cultured in DMEM supplemented with L-glutamine and 10% foetal calf serum whereas, retinal pigment epithelial (RPE-1) cells (Clontech) were cultured in DMEM-F12 supplemented with L-glutamine, sodium bicarbonate and 10% foetal calf serum (Invitrogen). Cells were transfected either with Lipofectamine (Invitrogen) or by calcium phosphate precipitation. COS were transiently transfected with 5 µg of DNA using lipofectamine plus reagent (Invitrogen) according to the protocol provided by the manufacturer. For immunofluorescent studies $4X10^6$ HeLa or RPE cells were electroporated with 20 µg of plasmid DNA in 500 µl of electroporation buffer (50 mM HEPES pH7, 100 mM NaCl in PBS) using a Gene Pulser II electroporator (Bio-Rad) with a capacitance of 975 µF and a voltage of 290V. Cells were plated out onto coverslips coated with 1 µg/µl fibronectin (Sigma) and 5 µg/µl collagen and fixed at various time points with -20^oC methanol.

Antibodies

The following antibodies were used either for immunofluorescent staining or western blotting purposes: anti-RbAp46 (AbCam), anti-CHD3 (Orbigen), anti-CHD3 (gift from W. Wang) anti-CHD4 (Orbigen), anti-CHD3/4 and anti-MTA2 (gifts from P. Wade). Secondary antibodies for immunofluorescent staining were obtained from the following sources: anti-mouse alexa 488, anti-mouse AMCA and anti-rabbit alexa 488 (all from

Molecular Probes); anti-mouse Cy3, anti-rabbit Cy3 and anti-human Cy5 (all from Jackson Immunochemicals). Horse radish perixoidase (HRP) linked anti-mouse and anti-rabbit secondary antibodies for western blotting purposes were obtained from Amersham.

Immunoprecipitations and Western blotting

Prior to lysis, cells were briefly washed with PBS, placed on ice and excess medium removed by aspiration. Cells were lysed in either in a low stringency buffer (50 mM Tris HCl pH7.5, 150 mM NaCl, 10 mM Na$_2$HPO$_4$, 1 mM EDTA pH8, 1% triton X-100) or a high stringency buffer (50 mM Tris HCl pH7.5, 150 mM NaCl, 1% sodium deoxycholate, 1% Triton X-100, 0.1%SDS). Lysates were cleared by centrifugation at 14, 000 rpm for 10 minutes at 4°C and then added to antibodies which had been incubated with 25 µl of protein A/G beads (Santa Cruz) for 30 minutes on ice. Proteins were immunoprecipitated overnight at 4°C with gentle mixing, the beads washed 5 times with low stringency lysis buffer and re-suspended in 50 µl of 2X SDS sample buffer. Proteins were denatured at 90°C for 3 minutes, fractionated on SDS polyacrylamide gels and transferred to Immobilon membrane (Amersham Pharmacia). Membranes were blocked with 01.% Tween-20, 5% non-fat dried milk in PBS for 1 hour at room temperature and incubated with primary antibodies diluted either in the same buffer or 5% BSA, PBS, 0.1% Tween-20 for 2 hours at ambient temperature or overnight at 4°C. Blots were washed 4 times with 0.1% Tween-20, 5% non-fat dried milk in PBS, incubated with secondary antibodies diluted in the same buffer for 1 hour at ambient temperature and washed 3 times with 0.2% Tween-20 in PBS. Revelation of blots was carried out using enhanced chemiluminescent development (KPL).

Immunofluorescence staining

Cells were grown on 12 mm acid washed circular coverslips and fixed with either -20°C methanol or 3.7% paraformaldehyde for 10 minutes at room temperature. Fixed cells were rehydrated by sequentially washing in PBS and then with a buffer consisting of PBS, 1% bovine serum albumin (BSA), 0.5% Triton X-100 (PBSA). Antibodies were diluted in PBSA, pipetted onto the surface of the coverslip and incubated at room temperature for 1 hour. The cells were washed with PBSA, secondary antibodies diluted in the same buffer added and incubated for 30 minutes at room temperature. DNA was stained using 1 μg/ml DAPI (Sigma).

siRNA

The following duplexes were synthesized for the purpose of gene silencing: CHD3, AAGCGUGACAGUGAGGAGGAA and AAGGCCAUCGAUCGGUUUAAU; CHD4, AAGGAUGAUGAUGAUGAUGAU and AACAGUUACCAAGAAGACUUA ; MTA2, AACCGGUAUAUUCAGCAGAAA; lamin AACUGGACUUCCAGAAGAACA (Dharmacon). Cells were transiently transfected with siRNA at a final concentration of 200 nM using oligofectamine (Invitrogen) according to the protocol provided.

Immunofluorescent microscopy and live cell imaging

Images of cells were taken using a Lecia microscope equipped a 100X objective lens and a cool-snap camera. Deconvolution was carried out using Metamorph software. Live cell

imaging was performed in 35 mm glass bottomed dishes overlaying medium with mineral oil (Sigma). A heated chamber (Harvard Apparatus) perfused with CO_2 was used to maintain a temperature of $37^{o}C$ and constant pH. Images were taken every 5 minutes with an Olympus microscope fitted with a 20X objective lens and an optibar.

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FIGURE LEGENDS

Figure 1 Components of the NuRD complex interact with pericentrin.

A) Yeast strain AH109, transformed with the C-terminus of either CHD3 (residues 1566-1966) or CHD4 (residues 1577-1912) or a C-terminal (C-ter) region of pericentrin A (peri, residues 1340-1756) grown on selective media to demonstrate interactions. DBD, DNA binding domain, TAD, trans-activation domain. **B**) COS cells co-transfected with HA-pericentrin (HA-peri) and FLAG-CHD4 expression constructs. Anti-FLAG or anti-GFP (control, Con) were used for immunoprecipitations (IPs). IPs were immunoblotted (IB) with antibodies as indicated. **C**) Co-IP of endogenous pericentrin with FLAG-CHD4 from transfected COS cell lysates. **D**) Immunoblots with antibodies to CHD3, CHD4, MTA2, RpAp46, MBD3 and FLAG after IP of FLAG-tagged pericentrin expressed in COS cells. **E**) CHD3/4 was immunoprecipitated from HeLa cell lysates and immunoblotted for pericentrin or CHD3/4.

Figure 2 NuRD components localize to centrosomes and midbodies

RPE-1 cells at the indicated cell cycle stages were stained with antibodies against NuRD components (green), a centrosomal marker (γ-tubulin or human autoimmune serum 5051, red) and DAPI (DNA, blue). Antibodies to all NuRD components except RbAp46 stained centrosomes of interphasic cells. CHD3 and MTA2 centrosomal staining was observed at all mitotic stages, whereas CHD4 appear to dissociate from the centrosome during mitosis. Central spindle and midbody staining during anaphase and telophase was also observed.

Figure 3 Over-expression of the C-terminus (CT) of CHD3 or CHD3 induces loss of pericentrin from centrosomes in interphase cells

A) HeLa cells electroporated with myc-tag constructs expressing the C-terminus of CHD3 or CHD4 or a control plasmid (GFP) were plated onto fibronectin/collagen-coated coverslips, fixed in -20°C methanol 6 hrs later and stained with anti-myc to detect overexpressed protein (red) or pericentrin (green). In most cases, pericentrin was either undetectable (at arrows) or reduced at centrosomes (boxes) in cells expressing CT-CHD3 or CT-CHD4 compared with control GFP-expressing cells (row 1) or non-transfected cells (column 2). In some cases, over-expressed CT-CHD4 localized to the centrosome (left cell). Insets, enlargements of boxes. **B**) Quantification of results from A show that many CT-CHD3/4 expressing cells have no detectable pericentrin (none, ~40%) or dramatically reduced levels (low). Low, staining intensity that is 50% of the brightest or below, high, 50-100% intensity levels.

Figure 4 RNAi-mediated depletion of CHD3 but not CHD4 disrupts centrosome integrity

A) RPE-1 cells treated with siRNAs targeting CHD3, CHD4 or lamin (control) for 72 hrs were lysed and immunoblotted (IB) for the indicated proteins. Note specific reduction of the targeted protein, but not others. Pericentrin levels unaffected under all conditions. **B**) Cells treated with siRNAs targeting CHD3 or CHD4 were fixed in -20°C methanol and stained with antibodies to CHD3, CHD4 or 5051 (centrosome marker). In many cells (~80%), CHD3 and CHD4 were depleted from nuclei and centrosomes. In cells deleted

of CHD3 a decrease in 5051 autoantibody staining was observed (e.g. left middle panel, bottom), suggesting a loss of many of the proteins known to react with this human autoimmune sera. In contrast, depletion of CHD4 did not show significant loss of 5051 staining (e.g. right middle panel, bottom).

Figure 5 CHD3 depletion reduces centrosomal levels of pericentrin and γ-tubulin but not centrin

A) HeLa cells treated with siRNAs against CHD3, CHD4 or lamin A/C (control) for 48 hrs were fixed in -20°C methanol and stained with antibodies to CHD3/4 (red) and pericentrin (green). Silencing of CHD3 (left) but not CHD4 or lamin (middle, right) induced loss of centrosomal pericentrin. Semi-quantitative analysis shows centrosomal staining intensity profiles (below images). Warmer colors and higher peaks represent greater signal intensities; circled areas next to numbers represent intensity profiles of corresponding centrosomes boxed and numbered in immunofluorescence images in panels above. **B**) HeLa siRNA-treated cells from experiment in A were stained with antibodies to CHD3 or CHD4 (red) and γ-tubulin (green). Insets, enlarged images of centrosomal γ-tubulin. Intensity profiles for γ-tubulin (see A). **C**) RPE-1 cells treated with siRNAs as in A for 72 hrs were fixed and stained with antibodies to CHD3 or CHD4 (red) and centrin-1 (green) to label centrioles. Insets, enlarged images of centrin-1 staining. Centrin-1 staining was similar under all conditions.

Figure 6 Microtubule organization and nucleation is diminished in cells overexpressing or depleted of CHD3

A) RPE 1 cells were electroporated with myc-tagged CHD3 or CHD4 C-terminus or GFP as a control. Cells were fixed 6 hours after transfection and stained with anti-α-tubulin and anti-myc antibodies. Cells transfected with either the C-terminus of CHD3 or CHD4 possessed fewer and more disorganized microtubule arrays compared to GFP controls with 84%, in each case, exhibiting the phenotype shown. In contrast only 3% of GFP-transfected cells had a disorganized microtubule array. B) Time course of microtubule regrowth. Cells depleted of CHD3, CHD4 or lamin (control) for 40 hours were treated with nocodazole for 90 min, washed free of drug, fixed at 2, 5 and 10 minute time intervals and stained for microtubules (red), centrosomes (green) and DNA (blue). Note fewer centrosome-nucleated microtubules (column 1, middle panel) and the curved, unfocused microtubules (column 1, lower panel). One microtubule aster (right box, first column) is not associated with a centrosome. Percentages refer to number of cells having disrupted microtubule arrays.

Figure 7 CHD3 depletion induces mitotic spindle defects

A) HeLa cells treated with the indicated siRNAs for 48 hrs were fixed with -20°C methanol and stained with anti-α-tubulin (red) and 5051 (green, centrosomes) antibodies. CHD3 depletion (upper panels) resulted in poorly organized spindles, misaligned chromosomes and diminished of bundled microtubules compared with CHD4 and lamin A/C siRNA treated cells (lower panels). Also see supplementary Fig. 2. Insets, 5051-labeled centrosomes. **B**) Quantification of spindle defects in siRNA-treated cells. Defects,

which include abnormal prometaphase-like phenotype (specific for CHD3), monopolar-, tripolar- and half-spindles and spindles with lagging chromosomes, are ~60% in CHD3-depleted cells. n = 100-200 mitotic cells counted for each condition. **C**) γ-tubulin staining in CHD3 depleted mitotic HeLa cells was reduced on poles and spindles and increased in the cytoplasm when compared with CHD4- and lamin AC-depleted cells.

Figure 8 CHD3 depletion causes metaphase delay, mitotic failure and cytokinesis defects

Still images from time-lapse movies (see supplemental movies) of HeLa cells depleted of lamin A/C (**A,** control) or CHD3 (**B, C**). Image collection was initiated 24 hrs after siRNA treatment and continued for >22 hours. **A**) Successful mitosis in lamin A/C depleted cell. **B**) Mitotic CHD3 depleted cell (time 0) enters anaphase (2 hrs 45 min) and appears to complete telophase (4 hrs 00 min) but ultimately fails cytokinesis to become a binucleated cell (14 hrs 40 min). **C**) Another CHD3 depleted cell (arrowhead) enters metaphase (18 hrs 40 min) and exits mitosis without dividing (20 hours 40 min). **D**) Graph showing timing of individual cells from nuclear envelope breakdown (NEB) to anaphase onset: ~38 minutes in lamin A/C depleted cells (range of 15-110 mins) and 265 mins in CHD3 depleted cells (range 40-435 mins).

Figure S1 Mapping of the pericentrin-binding domain on CHD4 using the yeast two-hybrid system

A series of GAL4-TAD CHD4 deletion constructs including the pericentrin-interacting fragment identified in the yeast two-hybrid screen and a GAL4-DBD pericentrin

expression construct were used to transform the yeast strain AH109. Colonies were streaked onto selective media to test for an interaction and a region between residues 1687-1880 of CHD4 was identified. Comparison shows that this domain is conserved between CHD3 and CHD4 sharing 81% sequence identity and 92% similarity.

Figure S2 Disrupted spindles in CHD3 depleted cells

Forty-eight hours after CHD3 depletion, HeLa cells were stained with anti-α-tubulin (microtubules, red) and 5051 antibodies (spindle poles, green). Insets, enalrgements of spindle pole body staining. All spindles show reduced microtubule numbers.

Figure S3 Delay in microtubule regrowth in CHD3 siRNA-treated cells after nocodazole washout

Additional images of regrowing microtubules in CHD3 siRNA-treated cells fixed 5 minutes after nodcodazole washout. The CHD3 depleted cell in the upper left panel has fewer microtubules associated with centrosomes (in boxes), many non-centrosome-associated microtubules in the periphery, a large microtubule free region surrounding asters and a nucleus displaced from the cell center.

A

	DBD	DBD-Peri C-ter
TAD-CHD3 C-ter		
TAD-CHD4 C-ter		

B

	IPs			Lysates			
	FLAG	Con					
HA-Peri	+	-	+	+	+	-	+	+
FLAG-CHD4	-	+	+	+	-	+	+	+

HA blot

FLAG blot

C

	FLAG IPs	Lysates
FLAG-CHD4	- +	- +

Peri blot

FLAG blot

D

	FLAG IPs	Lysates
FLAG-CHD4	- +	- +

CHD3

CHD4

MTA2

RbAp46

MBD3

FLAG

E

	IPs		Lysate
	nIgG	CHD3/4	

CHD3/4 blot

Peri blot

Figure 1

	Interphase	Prophase	Metaphase	Anaphase	Telophase
CHD3					
DNA NuRD centro					
CHD4					
DNA NuRD centro					
MTA2					
DNA NuRD centro					
RbAp46					
DNA NuRD centro					

Figure 2

A

	Over-expressed protein	pericentrin	merge
GFP			
CT-CHD3			
CT-CHD4			

B

Percentage cells

pericentrin staining

- >2
- high
- low
- none

GFP (n=200) CT-CHD3 (n=200) CT-CHD4 (n=200)

Figure 3

A

B

Figure 4

A

CHD3 siRNA   CHD4 siRNA   lamin siRNA

CHD3/4   DNA/pericentrin/CHD3/4   CHD3/4   DNA/pericentrin/CHD3/4   CHD3/4   DNA/pericentrin/CHD3/4

B

CHD3 siRNA   CHD4 siRNA   lamin siRNA

CHD3   DNA/γ-tubulin/CHD3   CHD4   DNA/γ-tubulin/CHD4   CHD3   DNA/γ-tubulin/CHD3/4

C

CHD3 siRNA   CHD4 siRNA   lamin siRNA

CHD3   DNA/centrin/CHD3   CHD4   DNA/centrin/CHD4   CHD3/4   DNA/centrin/CHD3/4

Figure 5

A

MT defects

| myc-CHD3 84% (n=100) | myc-CHD4 84% (n=100) | GFP 3% (n=200) |

α–tubulin

DNA/α-tubulin/ exog. protein

B

MT defects

| CHD3 siRNA 63% (n=300) | CHD4 siRNA 24% (n=300) | lamin A/C siRNA 1% (n=300) |

2 min

5 min

10 min

Figure 6

Figure 7

Figure 8

C6

1577   1629

1629   1912

N1

C5

1577   1682

1687   1912

N2

C4

1577

1733

C3

1577   1783

1787   1912

N4

C2

1577   1828

1827   1912

N5

C1

1577   1881

1880   1912

N6

1682

Interacting domain

1880

Figure S1

CHD3 siRNA

DNA
centrosome
α-tubulin
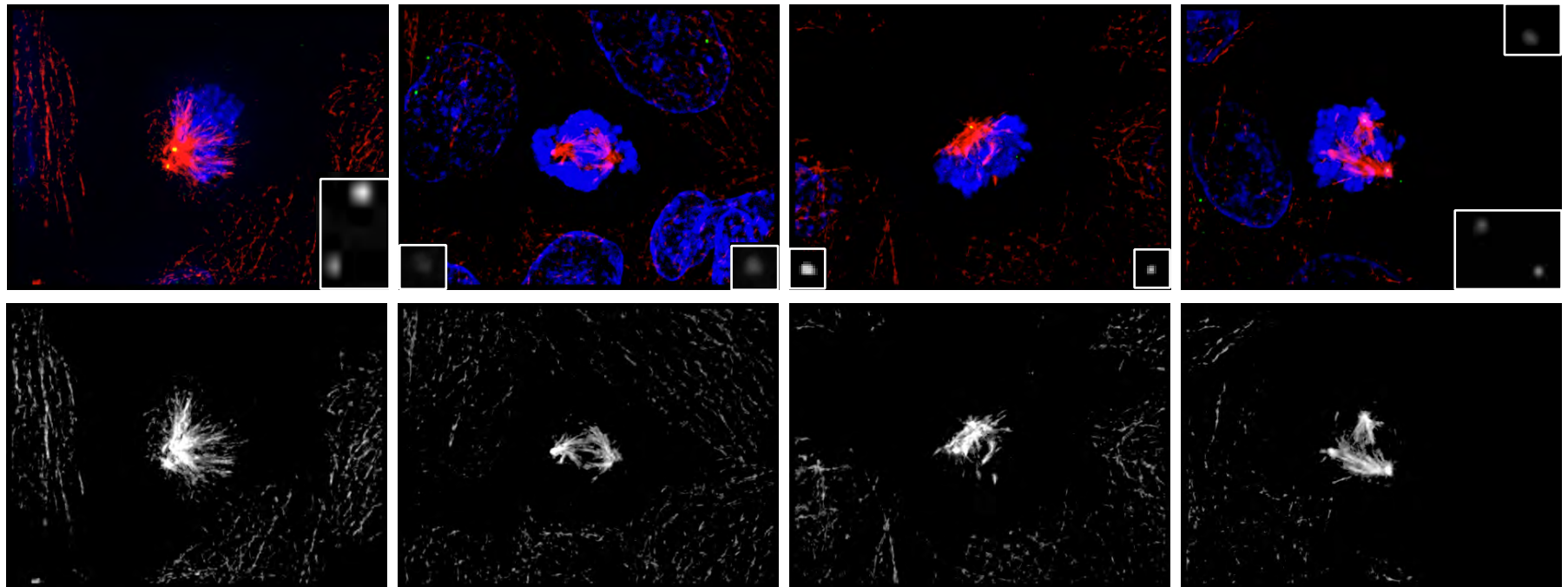
α-tubulin

Figure S2

CHD3 siRNA 5 minute microtubule regrow

DNA
centrosome
α-tubulin
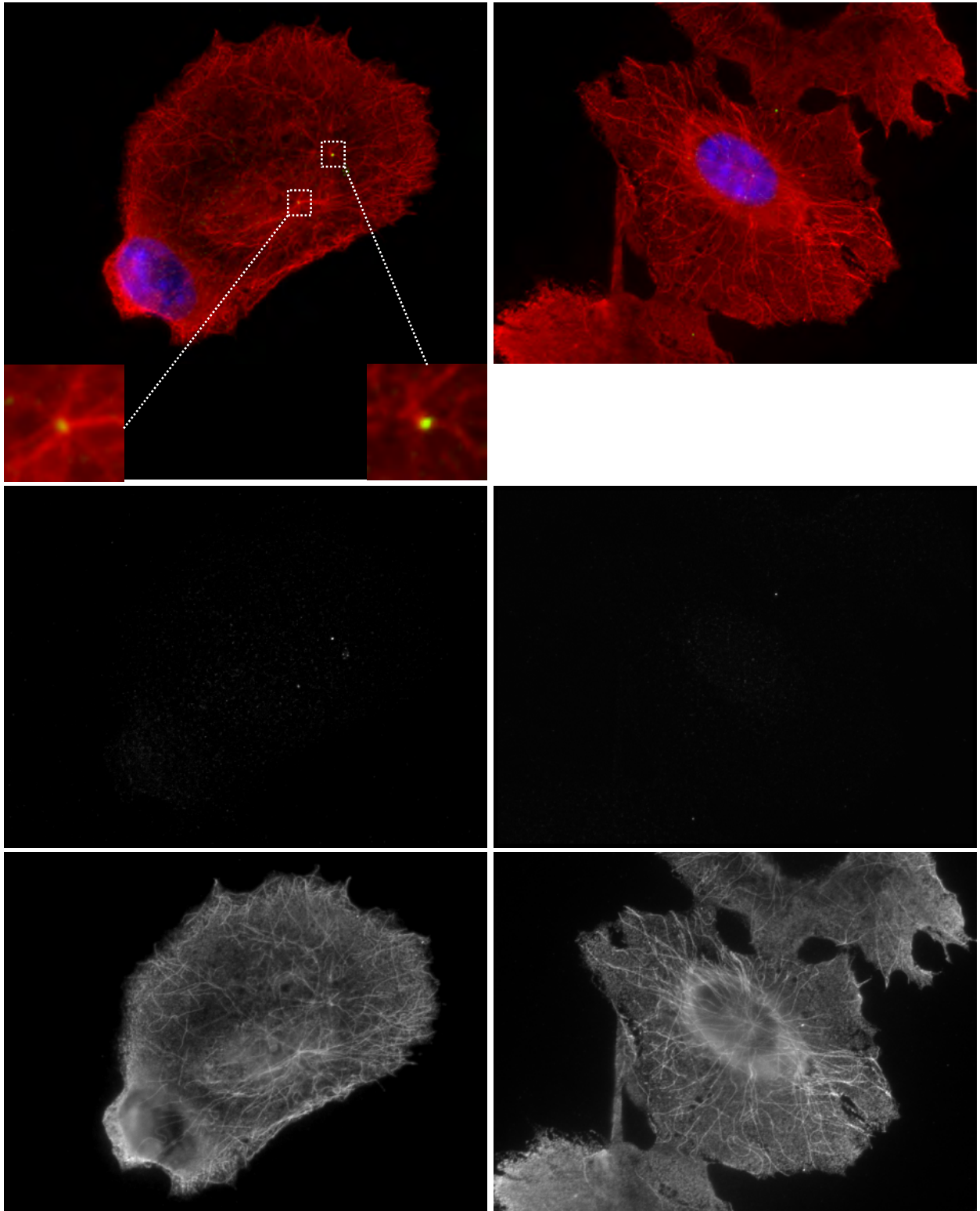
centrosome

α-tubulin

Figure S3

Mitosis-specific Anchoring of γ Tubulin Complexes by Pericentrin Controls Spindle Organization and Mitotic Entry

Wendy C. Zimmerman, James Sillibourne, Jack Rosa, and Stephen J. Doxsey*

Department of Molecular Medicine, University of Massachusetts Medical School, Worcester, Massachusetts 01605

Microtubule nucleation is the best known function of centrosomes. Centrosomal microtubule nucleation is mediated primarily by γ tubulin ring complexes (γ TuRCs). However, little is known about the molecules that anchor these complexes to centrosomes. In this study, we show that the centrosomal coiled-coil protein pericentrin anchors γ TuRCs at spindle poles through an interaction with γ tubulin complex proteins 2 and 3 (GCP2/3). Pericentrin silencing by small interfering RNAs in somatic cells disrupted γ tubulin localization and spindle organization in mitosis but had no effect on γ tubulin localization or microtubule organization in interphase cells. Similarly, overexpression of the GCP2/3 binding domain of pericentrin disrupted the endogenous pericentrin–γ TuRC interaction and perturbed astral microtubules and spindle bipolarity. When added to *Xenopus* mitotic extracts, this domain uncoupled γ TuRCs from centrosomes, inhibited microtubule aster assembly, and induced rapid disassembly of preassembled asters. All phenotypes were significantly reduced in a pericentrin mutant with diminished GCP2/3 binding and were specific for mitotic centrosomal asters as we observed little effect on interphase asters or on asters assembled by the Ran-mediated centrosome-independent pathway. Additionally, pericentrin silencing or overexpression induced G2/antephase arrest followed by apoptosis in many but not all cell types. We conclude that pericentrin anchoring of γ tubulin complexes at centrosomes in mitotic cells is required for proper spindle organization and that loss of this anchoring mechanism elicits a checkpoint response that prevents mitotic entry and triggers apoptotic cell death.

INTRODUCTION

The centrosome is the primary microtubule-organizing center in animal cells. At the centrosome core is a pair of barrel-shaped microtubule assemblies, the centrioles (Doxsey, 2001). Centrioles are capable of self-assembly (Marshall *et al.*, 2001; Khodjakov *et al.*, 2002) and can serve as templates for recruitment and organization of the surrounding pericentriolar matrix (Bobinnec *et al.*, 1998; Kirkham *et al.*, 2003). The pericentriolar material or centrosome matrix contains a high proportion of coiled coil proteins and is the site of microtubule nucleation. Within the matrix are large protein complexes of γ tubulin and associated proteins that have a ring-like structure and mediate the nucleation of microtubules called γ tubulin ring complexes or γ TuRCs (Moritz *et al.*, 1995a; Zheng *et al.*, 1995). Other proteins may share the ability to nucleate microtubules because centrosomes can organize microtubules in the absence of functional γ tubulin (Sampaio *et al.*, 2001; Strome *et al.*, 2001; Hannak *et al.*, 2002).

During cell cycle progression, centrosomes "mature" by recruiting additional γ TuRCs and several other proteins, resulting in an increase in the nucleation capacity of the centrosome (reviewed in Blagden and Glover, 2003). How-ever, we still know very little about proteins that directly anchor γ TuRCs to centrosomes in vertebrate cells. In the budding yeast, a small γ tubulin complex composed of γ tubulin (Tub4p), Spc97p, and Spc98p (~700 kDa) is bound to the nuclear side of the spindle pole body (the centrosome equivalent) through an interaction with Spc110p (Knop and Schiebel, 1997) and to the cytoplasmic side of the spindle pole body through Spc72p (Knop and Schiebel, 1998). Spc97p and Spc98p mediate binding of the complex to Spc110p and Spc72p (Knop and Schiebel, 1997; Knop and Schiebel, 1998; Nguyen *et al.*, 1998). Although there is no apparent homology between their SPC97/98 interacting domains, chimeras formed by fusing the binding domain of one with the localization domain of the other can rescue knockouts of the proteins encoding the localization domains, suggesting that the two binding domains are functionally homologous (Knop and Schiebel, 1998).

γ TuRCs in vertebrate cells and *Drosophila* contain orthologues of the three yeast proteins (γ tubulin and γ complex proteins 2 and 3 [GCP2, 3]) as well as several additional components (Zheng *et al.*, 1995; Martin *et al.*, 1998; Moritz *et al.*, 1998; Murphy *et al.*, 1998, 2001; Oegema *et al.*, 1999; reviewed in Job *et al.*, 2003). In vertebrates, the centrosome protein pericentrin (pericentrin A) forms a large complex with γ tubulin in the cytoplasm, and the two proteins are also in proximity at the centrosome (Dictenberg *et al.*, 1998). Recent evidence suggests there may be as many as 10 isoforms of pericentrin in human cells (Flory and Davis, 2003). A large isoform (pericentrin B/kendrin; Flory and Davis,

2003) and another centrosome protein called AKAP450/GC-NAP share homology with the calmodulin binding domain of Spc110p (Flory *et al.*, 2000; Gillingham and Munro, 2000; Li *et al.*, 2001). Other potential Spc110p orthologues have been identified in *Schizosacharomyces pombe*, *Aspergillus nudulans*, and *Drosophila* based on sequence homology (Flory *et al.*, 2002; Kawaguchi and Zheng, 2003) and in vertebrates (*Xenopus* and human) based on immunological cross-reactivity with Spc110p-specific antibodies (Tassin *et al.*, 1997). All proposed vertebrate orthologues of Spc110p localize to the centrosome and coimmunoprecipitate with γ TuRCs (Tassin *et al.*, 1997; Dictenberg *et al.*, 1998; Takahashi *et al.*, 2002). No Spc72p orthologues have been identified in other species.

In vertebrate cells, pericentrin B and AKAP450 have recently been shown to bind GCP2 in vitro (Takahashi *et al.*, 2002). Antibody inhibition and immunodepletion studies demonstrated a role for pericentrin isoforms and AKAP450 in microtubule nucleation in vertebrates and *Drosophila* (Doxsey *et al.*, 1994; Takahashi *et al.*, 2002; Kawaguchi and Zheng, 2003; Keryer *et al.*, 2003), perhaps by localizing the small Ran GTPase to centrosomes (AKAP450) (Keryer *et al.*, 2003). However, other studies show that antibody depletion of pericentrin B or reduction of pericentrin A and B do not affect aster formation, microtubule organization, or centrosome-associated γ tubulin (Li *et al.*, 2001; Takahashi *et al.*, 2002; Dammermann and Merdes, 2002). Moreover, loss of AKAP450 from centrosomes does not affect centrosomal γ tubulin localization, even though microtubule organization is disrupted (Keryer *et al.*, 2003). Another potential centrosomal γ TuRC-anchoring protein has recently been identified in vertebrate cells called ninein-like protein (Nlp), which can bind γ TuRC complexes, inhibit nucleation when neutralized with antibodies, and enhance nucleation when overexpressed (Casenghi *et al.*, 2003). However, we know little about the role of these putative scaffold proteins in centrosomal anchoring of γ TuRCs during the cell cycle and the cellular consequences of specifically disrupting their interactions with γ TuRCs at centrosomes.

In this study, we show that siRNAs targeting both pericentrin isoforms (A and B) induced specific loss of γ tubulin from spindle poles in mitosis, reduction of astral microtubules, and formation of monopolar spindles. This phenotype seemed to be specific for the smaller isoform of pericentrin because it was not observed when the larger pericentrin isoform was specifically reduced. A region at the C terminus of pericentrin interacted with both GCP2 and GCP3 in vitro as shown by coimmunoprecipitation and two-hybrid analysis. Expression of the GCP2/3 binding domain of pericentrin produced a phenotype similar to that observed in cells with reduced pericentrin. It disrupted the interaction between endogenous pericentrin and γ TuRCs, and adsorbed γ TuRCs from cell extracts. It reduced astral microtubules and centrosomal γ tubulin in mitotic cells and induced formation of small spindles and monopolar spindles. No effect on interphase microtubules was observed. When added to *Xenopus* extracts this domain dissociated γ tubulin from mitotic centrosomes and rapidly induced mitotic aster disassembly. The loss of γ tubulin from centrosomes in cells with reduced pericentrin levels or in cells expressing the GCP2/3 binding domain of pericentrin ultimately triggered a checkpoint inducing G2/antephase arrest and apoptosis in somatic cells. These phenotypes were not observed after specific reduction in the levels of the larger pericentrin isoform, expression of a mutant pericentrin defective in GCP2/3 binding, or expression of a homologous region of pericentrin B. We conclude that the smaller isoform of pericentrin provides a

molecular scaffold for centrosomal anchoring γ TuRCs during mitosis in both embryonic and somatic cell systems.

MATERIALS AND METHODS

Molecular Cloning

All pericentrin constructs used in this study were cloned into pcDNA vectors (Invitrogen, Carlsbad, CA) with amino terminal hemagglutinin (HA) tags (Purohit *et al.*, 1999; Purohit *et al.*, 2001), except those used in two-hybrid studies (see below). Fragments of pericentrin and other genes were polymerase chain reaction (PCR) amplified from cDNAs by using primers with *Not*I and *Xba*I restriction sites. PCR products were digested with the appropriate enzymes, cloned into the vector, and sequences were confirmed. In some cases, EcoR1 and *Xho*I restriction sites were used (peri B1826-2117, 1572-1816, 1572-1816m). GCP2-, GCP3-, and γ tubulin-containing constructs were obtained from Dr. Tim Stearns (Stanford University, Stanford, CA).

Small Interfering RNA (siRNA)

Twenty-one nucleotide RNAs were chemically synthesized by Dharmacon Reasearch (Lafayette, CO). and introduced to cells using Oligofectamine (Invitrogen, Carlsbad, CA.) in accordance with the manufacturer's instructions. The target sequences used were AAUUGGAACAGCUGCAGCAGA against pericentrin A and B in human (Dammermann and Merdes, 2002), AAUGAGGGUGUCCACAGGAGA against pericentrin A and B in mouse, and AAGCUCUGAUUUAUCAAAAGA against the PACT domain of pericentrin B in human. AACUGGACUUCCAGAAGAACA, which targets human lamin A and is nonspecific in mouse, was used as a control for all siRNA studies. Crude cell lysates were analyzed for protein silencing. Cells were treated with 2 mM thymidine for 18 h starting 24 h post-siRNA treatment. Six hours after thymidine release, cells were harvested and lysed in phosphate-buffered saline (PBS) supplemented with 1% Triton X-100, 10 μg/ml leupeptin, 10 μg/ml pepstatin, 10 μg/ml chymotripsin,10 μg/ml phenylmethylsulfonyl fluoride, 2.0 μg/ml *p*-amino-benzamidine, 5 mM iodoacetamide, and 5 mg/ml *N*-ethylmaleimide. Cell lysates were clarified at top speed in a Microfuge for 15 min at 5°C. Protein concentration for each lysate was determined using Bio-Rad protein dye reagent, loads were adjusted, proteins were resolved by SDS-PAGE, and analyzed by Western Blot.

Antibodies

Anti-myc, anti-γ-tubulin, and anti-tubulin antibodies were obtained from Sigma-Aldrich (St. Louis, MO). Phosphohistone H3 rabbit polyclonal antibody was purchased from Upstate Biotechnology (Lake placid, NY). M30 Cytodeath and anti-HA rat monoclonal antibody 3F10 was obtained from Roche Diagnostics (Indianapolis, IN) Anti-human lamin A/C antibody was purchased from Cell Signaling Technology (Beverley, MA). Other antibodies included M8 anti-pericentrin antibody, (Dictenberg *et al.*, 1998), human autoimmune serum 5051 that recognizes centrosome proteins (Doxsey *et al.*, 1994), anti-pericentrin B/kendrin-specific antibody (Flory et.al., 2000) (obtained from Trisha Davis, University of Washington, Seattle, WA), anti-GCP2 antibody (Murphy *et al.*, 1998) (obtained from Dr. Tim Stearns), and anti-GCP3 antibody (a gift from Michel Bornens, Institut Curie, Paris, France).

Yeast Two-Hybrid Cloning/Methods

Direct yeast two-hybrid interactions were performed essentially as described previously (Gromley *et al.*, 2003). Pericentrin, γ tubulin, GCP2, and GCP3 coding sequences were amplified from plasmid DNA by PCR by using Pfu Turbo (Stratagene, La Jolla, CA), cloned into either pGBKT7 or pGADT7 (BD Biosciences Clontech, Palo Alto, CA) and completely sequenced. Yeast strains AH109 and Y187 were transformed with GAL4 DNA binding domain (GAL4-DBD) or GAL4 transactivation domain (GAL4-TAD) expression constructs, respectively, and diploid strains generated by mating. Interactions between pericentrin and members of the γ tubulin ring complex were tested for by streaking yeast onto synthetic defined (SD) medium lacking leucine, tryptophan, histidine, and adenine.

Biochemical Techniques

Immunoprecipitations from *Xenopus* extracts were performed as described previously (Dictenberg *et al.*, 1998) by using the antibodies to the pericentrin amino terminus (M8) (Doxsey *et al.*, 1994) and γ tubulin (Zheng *et al.*, 1995). For disruption of γ TuRCs from pericentrin in coimmunoprecipitations, active or heat denatured pericentrin fractions were added directly to *Xenopus* high-speed extracts before immunoprecipitation. Protein affinity experiments to recruit γ TuRCs (Figure 2) were performed using partially purified fractions of pericentrin domains (see below). Proteins were bound to anti-HA beads, added to extracts for 60 min, washed in extract buffer (Murray, 1991), run on SDS gels, and probed with the indicated antibodies.

Proteins for recruitment of γ TuRCs (Figure 2) and for aster inhibition assays (Figures 4 and 5) were produced in COS cells and purified as follows. Confluent COS cells were transiently transfected with 3 μg of DNA/60-mm
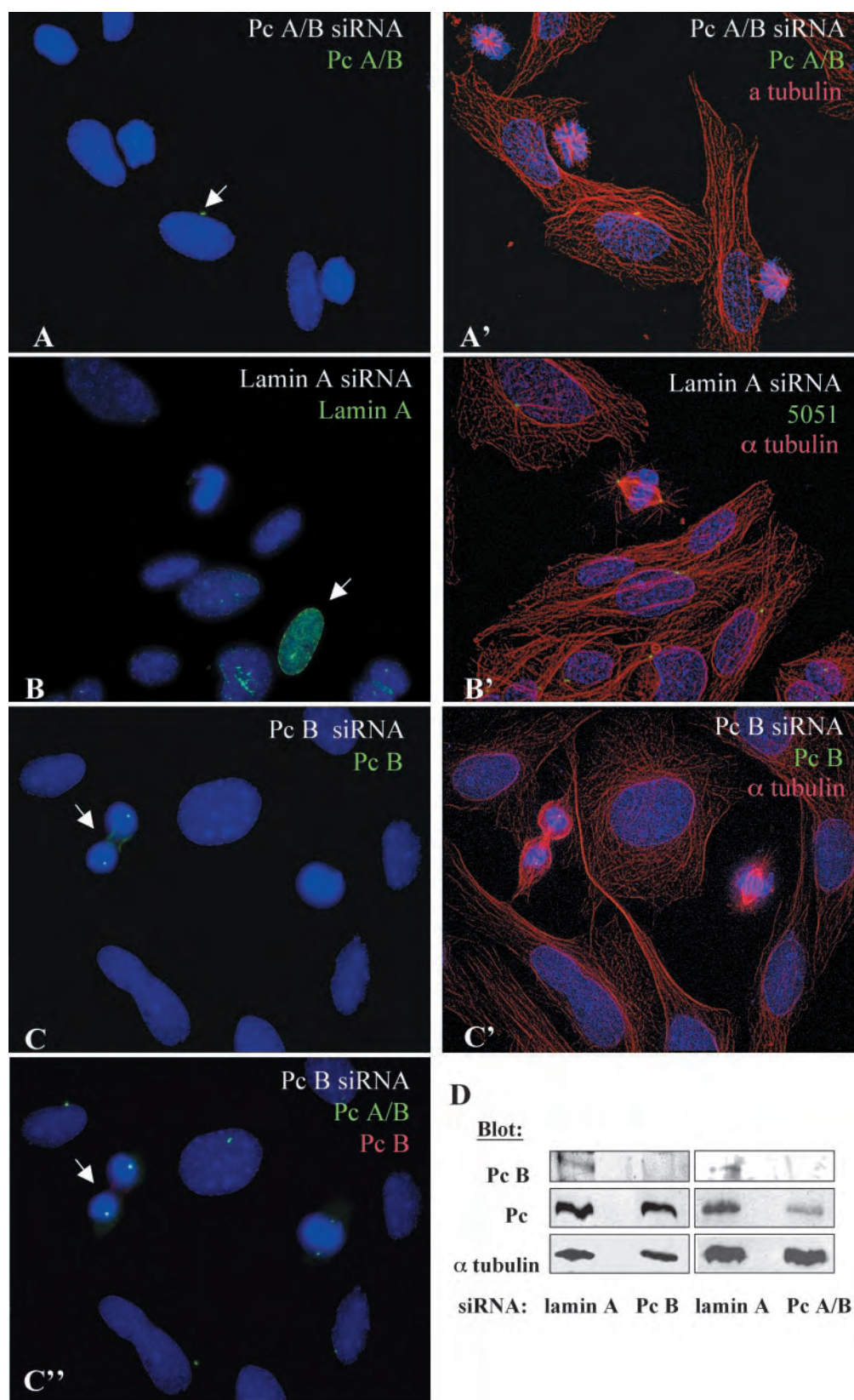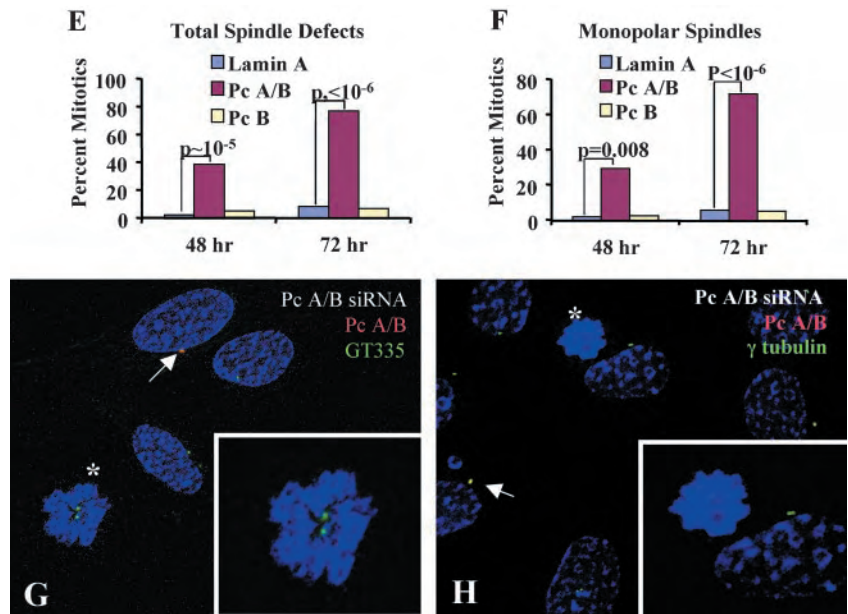
Figure 1.

Figure 1 (cont). Silencing of pericentrin A and B causes mitotic defects. (A) SAOS cells with reduced pericentrin after siRNA treatment stained with a pericentrin antibody (M8, green), which recognizes both isoforms of pericentrin (Pc A/B) and DNA (DAPI, blue). (A′) Same field as in A costained for microtubules (α tubulin, red). (B) Cells with reduced lamin A stained for lamin A (green) and DNA. (B′) Same field as in B stained for centrosomes with 5051 autoimmune sera (green) and microtubules (red). (C and C′) Cells with reduced pericentrin B (targeting the C-terminal PACT domain) stained with pericentrin B-specific antibody, Pc B, (C, green), together with microtubule label (C′) or pericentrin antibody that recognizes both isoforms (C″). (A, B, C, and C″) Maximum projection of z series without deconvolution. (A′, B′, and C′) Maximum projection of deconvolved z series. Note the improvement in the resolution of the DNA. Arrows indicate cells expressing near normal levels of the targeted protein. (D) Western blots of crude cell lysates demonstrating reduction of pericentrin B (Pc B) but not pericentrin A (Pc) by using Pc B-specific siRNA or reduction of both isoforms relative to lamin A siRNA control, by siRNA targeting Pc A and Pc B (Pc A/B). Pericentrin isoforms probed with Pc A/B anti-pericentrin antibody (M8). α Tubulin loading control probed with DM1α anti-alpha tubulin antibody. (E) Graph showing percentage of mitotic SAOS cells with spindle defects (monopolar, multipolar, and reduced astral microtubules) at 48 and 72 h after siRNA treatment. One hundred to 150 mitotic cells scored per bar. (F) Graph indicating percentage of mitotic cells with monopolar spindles at 48 and 72 h. (G) Cells with reduced pericentrin A/B (Pc A/B, red) retain the centriole marker GT335 (green) in interphase and mitosis. (H) γ Tubulin in cells with reduced pericentrin A/B seems largely unchanged at centrosomes in interphase but is reduced at spindle poles. Pc A/B (red), γ tubulin (GTU88, green), and DAPI (blue). (G–H) Maximum projection of z series with no neighbor deconvolution. Arrows indicate cells with near normal staining levels of pericentrin. Asterisks indicate mitotic cells. Mitotic cells are show at higher magnification in insets.

dish by using LipofectAMINE Plus reagent (Invitrogen). Transfected cells were maintained for 3 d in DMEM with 5% serum and then collected with 5 mM EDTA in PBS. Cells were lysed in PBS supplemented with protease inhibitor cocktail (#1836153; Roche Diagnostics, Basel, Switzerland), 1% Triton X-100, and 5 mg/ml N-ethylmaleimide. For recruitment of γ tubulin from extracts, HA beads were prepared by pretreating Dynabeads 450 (#110.05; Dynal, Lake Success, NY) with a saturating amount of anti-HA antibody 12CA5 (Covance, Denver, PA). Anti-HA IgG beads were treated with COS cell lysate containing an excess of the indicated HA-tagged pericentrin polypeptides, washed three times in PBS lysis buffer, two times in PBS, and two times in extract buffer, before addition of *Xenopus* extracts for γ TuRC recruitment experiments. For preparation of soluble HA-tagged pericentrin, 12CA5 antibody was cross-linked to protein A beads (Bio-Rad, Hercules, CA) by using standard methods (Harlow and Lane, 1988). HA-tagged pericentrin was batch depleted from COS lysates by incubation with HA cross-linked beads at 5°C with gentle agitation for 1 h. Treated beads (configured as a column) were washed with 10 column volumes of lysis buffer, 10 volumes of PBS with protease inhibitors, and 10 volumes of 10 mM Tris, pH 8.0. HA-tagged pericentrin was eluted with 2 volumes of 150 mM glycine, pH 2.5, into 1/4 volume of 1 M Tris, pH 8.0, and dialyzed against PBS overnight.

Coimmunoprecipitations

Coimmunoprecipitation of pericentrin isoforms and γ TuRC components (Figure 3) was performed in COS cells 40–48 h after transient cotransfection of the indicated constructs by using LipofectAMINE Plus reagent. Cells were collected using 5 mM EDTA in PBS. Cell pellets were lysed with 1% NP-40, 1 mM dithiothreitol, 10% glycerol in buffer C (100 mM PIPES, pH 6.9, 6 mM MgCl$_2$, 0.5 mM EGTA, 10 μg/ml leupeptin, 10 μg/ml pepstatin, 10 μg/ml chymotripsin, 10 μg/ml phenylmethylsulfonyl fluoride, 2.0 μg/ml p-aminobenzamidine, 5 mM iodoacetamide). Lysates were clarified 15 min at top speed in a Microfuge at 5°C and then applied to HA Dyna beads (see above). Beads were treated for 1 h at 5°C with end-over-end agitation and washed two times in lysis buffer (see above) and two times in wash buffer (buffer C with 100 mM Na acetate, pH 6.9). Loads and treated beads (immunoprecipitates) were analyzed by SDS gel electrophoresis and Western blot by using the indicated antibodies.

Xenopus Extracts

Cytostatic factor (CSF)-arrested *Xenopus* extracts were prepared, and aster assembly assays were preformed as described previously (Murray, 1991;

Stearns and Kirschner, 1994). For purpose of quantization, two hundred sperm were counted and scored for the presence of assembled microtubules. In some cases, the standard fix [0.3 volume of 37% formaldehyde, 0.6 volumes of 80% (wt/vol) glycerol, 0.1 volume 10× MMR, 1 μg/ml 4,6-diamidino-2-phenylindole (DAPI)] was modified by the addition of 0.05% Oligreen (Molecular Probes, Eugene, OR) to facilitate visualization of sperm nuclei with a scanning confocal microscope. Centrosome assembly in the presence of nocodazole was preformed using published methods (Stearns and Kirschner, 1994). Treated nuclei were prefixed in 5% formaldehyde, spun onto coverslips through a 20% sucrose cushion by using a JS13.1 rotor at 8000 rpm 15 min, and postfixed in methanol (−20°C) before staining for immunofluorescence. Ran-mediated asters were prepared using constitutively active RanL43E as described previously (Wilde and Zheng, 1999). Centrosome-dependent and -independent *Xenopus* mitotic asters were fixed in formaldehyde on coverslips as described previously (Murray, 1991; Wilde and Zheng, 1999).

Cell Lines

Cell lines (COS-7, SAOS, and U2OS) were grown as described previously (American Type Culture Collection, Manassas, VA) and prepared for transfection experiments as described previously (Purohit *et al.*, 1999). Primary mouse embryonic fibroblasts (MEFs) were obtained from Dr. Geoffrey Wahl (Salk Institute for Biological Studies, La Jolla, CA) and used at less than six passages.

Transfection and Immunofluorescence

For transfection and immunofluorescence analysis, logarithmically growing cells were transfected as indicated by the manufacturer with 1 μg of DNA/35-mm dish of the appropriate construct by using LipofectAMINE Plus (Invitrogen) for COS cells and LipofectAMINE for SAOS and U2OS cells. The transfection efficiency for COS cells with control constructs ranged from 35 to 60%. MEFs were transfected using Superfect (QIAGEN, Valencia, CA). For immunofluorescence, cells were fixed with –20°C MeOH as described previously (Purohit *et al.*, 1999). Data were collected as a Z series for deconvolution with 0.3 μm between planes. Images were deconvolved using MetaMorph software, no neighbors algorithm. All images were rendered two dimensional by showing maximum intensity at each point.

Microinjection Experiments

For microinjection, COS cells were synchronized by thymidine block. Cells were treated 16 h with 2 mM thymidine and released (single block), or treated

for an additional 16 h with 2 mM thymidine after 8 h of release (double block). The mitotic index of synchronized cells was determined using replicate coverslips, fixed and stained with DAPI, and then counted at the indicated times postrelease from thymidine block. 1000 cells were counted for each time point. Microinjection into the nucleus of released cells was performed using an Eppendorf transjector 5246, with Eppendorf femtotips, with an injection pressure of 100 hPa, injection time of 0.4 s, and DNA at a concentration of 0.2 μg/μl in PBS.

RESULTS

Pericentrin Silencing Mislocalizes γ Tubulin from Spindle Poles and Disrupts Spindle Bipolarity

We previous showed that pericentrin interacts with the γ tubulin ring complex and that pericentrin antibodies disrupt spindle organization and function (Doxsey *et al.*, 1994; Dictenberg *et al.*, 1998). In this study, we address the molecular mechanism of the mitotic function of pericentrin. Initially, we used siRNAs designed to silence the two previously characterized isoforms of pericentrin (A and B/kendrin), although we cannot rule out silencing of other potential pericentrin isoforms under these conditions (Flory and Davis, 2003). We typically observed silencing in 80 to 90% of treated cells (Figure 1, A–C, G, and H). Silencing of pericentrin A/B disrupted mitotic spindle organization and reduced astral microtubules, ultimately leading to the formation of monopolar spindles in most mitotic cells (Figure 1, A, A', E, and F). The phenotype seemed to be specific for mitotic cells because interphase microtubule organization was not detectably altered (Figure 1, A and A'). γ Tubulin was reduced at spindle poles in mitotic cells, although centrioles were present; centrosomes in adjacent interphase cells retained strong γ tubulin staining (Figure 1, G and H). Selective silencing of the larger isoform of pericentrin (B) had no effect on interphase or mitotic microtubule organization (Figure 1C, C', C", and D), although we cannot rule out the possibility that activity of the residual protein is sufficient to support these functions. These results suggested that the phenotype observed after pericentrin A/B silencing resulted from reduction in pericentrin A, although this isoform could not be specifically targeted because it is homologous through most of its length with pericentrin B (Flory and Davis, 2003). Control cells with reduced lamin levels showed no detectable changes in any of the parameters described above (Figure 1, B, B', E, and F; our unpublished data).

Pericentrin Interacts with the γ TuRC in Xenopus Extracts through GCP2 and 3

We next examined the relationship of pericentrin and the γ tubulin ring complex in more detail. We found that immunoprecipitation of pericentrin from *Xenopus* extracts coprecipitated several components of the γ TuRC, including γ tubulin, GCP2, and GCP3 (Figure 2A). Conversely, immunoprecipitation of γ tubulin coprecipitated pericentrin in addition to GCP2 and GCP3. Additional evidence for the pericentrin-γ TuRC interaction was obtained by showing that an HA-tagged C-terminal region of pericentrin affixed to beads could be used to specifically pull out endogenous γ tubulin and associated proteins from *Xenopus* extracts (Figure 2B, 1340–1920; our unpublished data). Moreover, the C-terminal region of pericentrin was able to disrupt the endogenous pericentrin–γ TuRC interaction when added to extracts as shown by the loss of γ tubulin from pericentrin immunoprecipitates (Figure 2C). These results demonstrate that pericentrin interacts with the γ TuRC and that the interaction is mediated by a domain at the C-terminal region of the protein.

Figure 2. Pericentrin interacts with the γ TuRC in *Xenopus* extracts. (A) Immunoprecipitation of endogenous pericentrin pulls down γ TuRC proteins (γ tubulin, GCP2, and GCP3) from *Xenopus* extracts (lane 2) and immunoprecipitation of γ tubulin pulls down pericentrin (lane 3), whereas nonspecific rabbit IgG precipitates none of these proteins (lane 1). (B) HA-tagged C-terminal domains of pericentrin bound to anti-HA beads pull down endogenous γ tubulin from *Xenopus* extracts (lanes 4 and 5), whereas beads alone and HA-tagged central and amino-terminal domains do not pull down significant γ tubulin (lanes 1–3). (C) A C-terminal domain of pericentrin (1618–1810) disrupts the interaction between endogenous pericentrin and the γ TuRC in extracts as shown by immunoprecipitation with anti-pericentrin antibodies, whereas heat-inactivated protein (h.i. 1618–1810) and phosphate-buffered saline (PBS) have no effect. Numbers in B and C represent amino acid numbers of pericentrin.

Figure 3. C-terminal domains of pericentrin interact with γ TuRC proteins GCP3 and GPC2 in vitro. (A) When coexpressed in vertebrate cells myc-tagged GCP2 and/or myc-tagged GCP3 coimmunoprecipitate with HA-tagged pericentrin (similar mobility of GCP2 and GCP3 prevents their individual identification in this experiment). (B–D) A C-terminal domain of pericentrin (amino acids 1340–1920) interacts with GCP3 (B) and GCP2 (C) but not γ tubulin (D) when coexpressed in vertebrate cells. Immunoprecipitation and immunoblotting performed as indicated. End., endogenous protein. (E) Two-hybrid analysis confirms the interaction of the pericentrin C terminus with GCP2 and GCP3 but not with γ tubulin (data not shown). (F and G) Segments of pericentrin-B corresponding to the C terminal region of pericentrin do not interact with GCP2 in two-hybrid (F) or coimmunoprecipitation experiments (G).

To determine the molecular basis of the interaction of pericentrin with the γ TuRC, we tested whether pericentrin could bind individual proteins of the complex in vitro. We found that HA-tagged full-length pericentrin and the C-terminal third of pericentrin coimmunoprecipitated myc-tagged GCP3 when the proteins were cooverexpressed in COS-7 cells (Figure 3, A and B). In parallel assays, the pericentrin C terminus coimmunoprecipitated myc-tagged GCP2 (Figure 3, A and C) but not myc-tagged γ tubulin (Figure 3D). GCP2/3 binding was specific for the C terminus of pericentrin because several domains comprising the

amino terminal two-thirds of the molecule showed no interaction (Figure 3, B and C). Direct two-hybrid analysis confirmed the interaction of the pericentrin C terminus with both GCP2 and GCP3 (Figure 3E) and failed to detect an interaction with γ tubulin or amino terminal domains of pericentrin (our unpublished data). In addition, domains of the larger isoform (pericentrin B) that included the GCP2/3 interacting region of pericentrin as well as an additional exon not present in pericentrin (66% identical, 78% similarity) did not interact with GCP2 (or GCP3) by immunoprecipitation (Figure 3G) or two-hybrid analysis (Figure 3F, see

Figure 4. C-terminal fragments of pericentrin disrupt aster formation and stability and γ tubulin assembly onto centrosomes in *Xenopus* mitotic extracts. (A and B) Mitotic asters assembled in the presence of equal amounts of pericentrin 1340–1920 (B) or heat inactivated (h.i.) 1340–1920 (A). (C) Mitotic aster assembly in the presence of increasing concentrations of pericentrin (Pc) 1340–1920. (D) Quantification of aster assembly in mitotic extracts in the presence of pericentrin domains. Amount of protein added per 10 μl of extract is indicated. (1, PBS; 2, 33 ng of 1–535; 3, 12 ng of 1340–1920 (p < 0.0001); 4, 12 ng of h.i. 1340–1920; 5, 10 ng of peri B1826–2117; 6, 10,000 ng of BSA). Quantification of aster assembly in interphase extracts (D7–9) by using a pericentrin domain (1618–1810) that inhibits aster assembly in mitotic extracts (7. p < 0.0001) and is inactivated by heat (8), but has no activity in interphase extracts in the same experiment (9). (E and F) γ Tubulin assembly onto nascent centrosomes in the presence of h.i. 1340–1920 (E) or 1340–1920 (F, p < 0.0001). (G) Quantification of γ tubulin assembly onto centrosomes in the absence of mitotic extract (1), in extracts with 1–595 (2), 1340–1920 (3, p < 0.0001), 1618–1810 (4, p < 0.0001), or heat inactivated 1618–1810 (5). For C, D, and G, 200 sperm nuclei were counted per bar or point. (H–K) Rapid disassembly of preassembled mitotic asters over time after addition of 1340–1920. (L) h.i. Pc 1340–1920 has no effect on preassembled asters. (M and N) Ran-mediated aster assembly in extracts in the presence of h.i 1618–1810 (M) or 1618–1810 (N). A–D, H–N, microtubules or γ tubulin, red; nuclei, green. Bar (A), 10 μm for A and B; in L, 10 μm for H–N.

accession numbers gi458668 and gi31296687 for more details on sequence differences). Data from these two independent assays demonstrate that pericentrin interacts specifically with at least two members of the γ TuRC, GCP2 and GCP3. The C terminus of pericentrin seemed to bind GCP2 and GCP3 more efficiently than the full-length molecule. Similar binding patterns have been observed for other pericentrin-interacting proteins such as the dynein light intermediate chain, and they could result from increased accessibility to epitopes that are masked in the full-length protein (Tynan *et al.*, 2000).

The C Terminus of Pericentrin Disassembles Mitotic Asters and Centrosomal γ Tubulin in Xenopus Extracts

Microtubule aster formation on nascent centrosomes of sperm nuclei in *Xenopus* extracts is dependent on the recruitment of soluble γ TuRCs to these sites (Felix *et al.*, 1994; Stearns and Kirschner, 1994). Previous studies implicated pericentrin in this process (Dictenberg *et al.*, 1998; Doxsey *et al.*, 1994). To address this issue directly, we examined the effect of the GCP2/3 interacting domain of pericentrin on microtubule aster assembly in mitotic *Xenopus* extracts. Addition of this domain to extracts before the aster assembly reaction significantly reduced aster formation (Figure 4, A and B). Even after extended periods (30 min), few asters were detected, and they had few microtubules and were highly disorganized, a phenotype almost never observed in controls. Half maximal aster inhibitory activity was seen at a protein concentration ~4:1 with endogenous pericentrin (Figure 4C). No change in aster assembly was observed in the presence of the pericentrin N terminus, heat-denatured C terminus, bovine serum albumin, or buffer alone (Figure 4D, 1–6). The activity seemed to be specific for mitotic extracts as there was no detectable effect on aster assembly in interphase extracts (Figure 4D, 7–9).

The mechanism of aster inhibition was examined in more detail by monitoring recruitment of γ tubulin onto nascent centrosomes in *Xenopus* mitotic extracts as described previously (Doxsey *et al.*, 1994; Felix *et al.*, 1994; Stearns and Kirschner, 1994). The pericentrin C-terminal domain and subdomains of this protein specifically inhibited recruitment of γ tubulin onto centrosomes to the same extent and at the same concentration that prevented microtubule aster assembly and disrupted the interaction between pericentrin and the γ TuRC (Figure 4, E–G). These results suggested that the pericentrin C terminus inhibited microtubule aster formation in mitotic extracts by preventing recruitment of γ tubulin to pericentrin sites on the nascent centrosome.

To more directly test whether pericentrin anchored γ TuRCs to nascent centrosomes, we examined the effect of the pericentrin C-terminal polypeptide on asters preassembled in extracts. Within 60 s after addition of the protein, the focus of microtubules in preassembled asters was disrupted, and free microtubules were observed in the region surrounding the aster (Figure 4, H–K). By 2 min after addition of the protein, most microtubules seemed to have lost their attachment to the centrosome; the remaining microtubules were of normal length and often formed bundles. By 3–5 min, no microtubules were detected at most centrosomes. In contrast, preassembled asters exposed to heat-inactivated pericentrin C terminus (Figure 4L), other pericentrin domains, the pericentrin B homology domain, or buffer alone showed no detectable loss of centrosomal microtubules, no change in microtubule organization, and few to no free microtubules in the vicinity of the aster. Pericentrin C-terminal peptide was just as effective at disrupting preexisting asters as it was at inhibiting their assembly, through a range

of test concentrations. Pericentrin C terminal peptide caused loss of γ tubulin from preassembled centrosomes within the same time frame that it caused loss of microtubules from asters (90% reduction in 5 min). These results indicate that the pericentrin C terminus disrupts the interaction of γ TuRCs with centrosomes releasing the complexes and attached microtubules.

Microtubule asters can form in *Xenopus* extracts by a centrosome-independent pathway that requires the Ran GTPase (reviewed in Dasso, 2002). Ran-mediated aster assembly can be inhibited by a dominant negative form of Ran and enhanced by a dominant active form of the protein. Under conditions that resulted in rapid disassembly of mitotic asters, the pericentrin C terminus did not significantly affect Ran-mediated aster assembly even after extended periods of incubation (Figure 4, M and N). Thus, although formation of Ran asters requires γ tubulin (Wilde and Zheng, 1999), it seems to be less dependent on pericentrin than does centrosome-mediated aster assembly.

Mapping the GCP2/3 Binding Domain and Aster-disrupting Activity of Pericentrin

We further defined the pericentrin–GCP2/3 interaction site and made point mutants that inhibited the pericentrin–GCP2/3 interaction. Using directed two-hybrid and coimmunoprecipitation analyses, we identified a subdomain of the C terminus that was required for strong GCP2/3 binding in both assays (Figure 5A, consensus). We identified a point mutation in this domain with significantly reduced binding to GCP2 in vitro and lacked aster activity in *Xenopus* extracts. GCP2 and GCP3 bind cooperatively to pericentrin with in the consensus region because myc-tagged GCP2 showed cooperative binding to HA-tagged pericentrin in the presence of GCP3 (Figure 5C). In functional assays, pericentrin domains that bound GCP2/3 showed aster inhibitory activity in *Xenopus* extracts (Figure 5A). Those that did not interact with GCP2/3 lacked aster inhibitory activity, including the pericentrin mutant, a domain of pericentrin B containing all pericentrin sequences required for activity (Figure 5A), and several pericentrin domains outside the GCP2/3 interacting domain (Figure 5A, consensus). The strong correlation between regions of pericentrin that interacted with GCP2/3 and those that showed mitotic aster and γ TuRC-disrupting activity indicated that pericentrin was required for anchoring γ TuRCs to centrosomes in *Xenopus* mitotic extracts.

To further address differences in GCP2/3 binding between pericentrin (A) and pericentrin B, we excised most of an extra exon (and some additional sequences) that is present in the homologous region of pericentrin B. Truncated pericentrin B proteins lacking the amino acids encoded by these sequences had weak GCP2 binding activity (Figure 5, A and D), suggesting that pericentrin B binding to the γ TuRC in this region may be blocked by incorporation of an extra exon.

GCP2/3 Interacting Domains of Pericentrin Disrupt Mitotic Asters and Spindles in Vertebrate Cells

We next tested whether the GCP2/3-interacting domains of pericentrin affected microtubule organization in vertebrate cells. We found that these domains had no detectable effect on the organization or nucleation of microtubules or the organization of centrosomes in interphase SAOS cells (Figure 6A). However, the same domains disrupted microtubule structures in mitotic cells (Figure 6, B–K). The most common phenotype was monopolar spindles, which represented ~15% of all mitotic cells at early times posttransfection
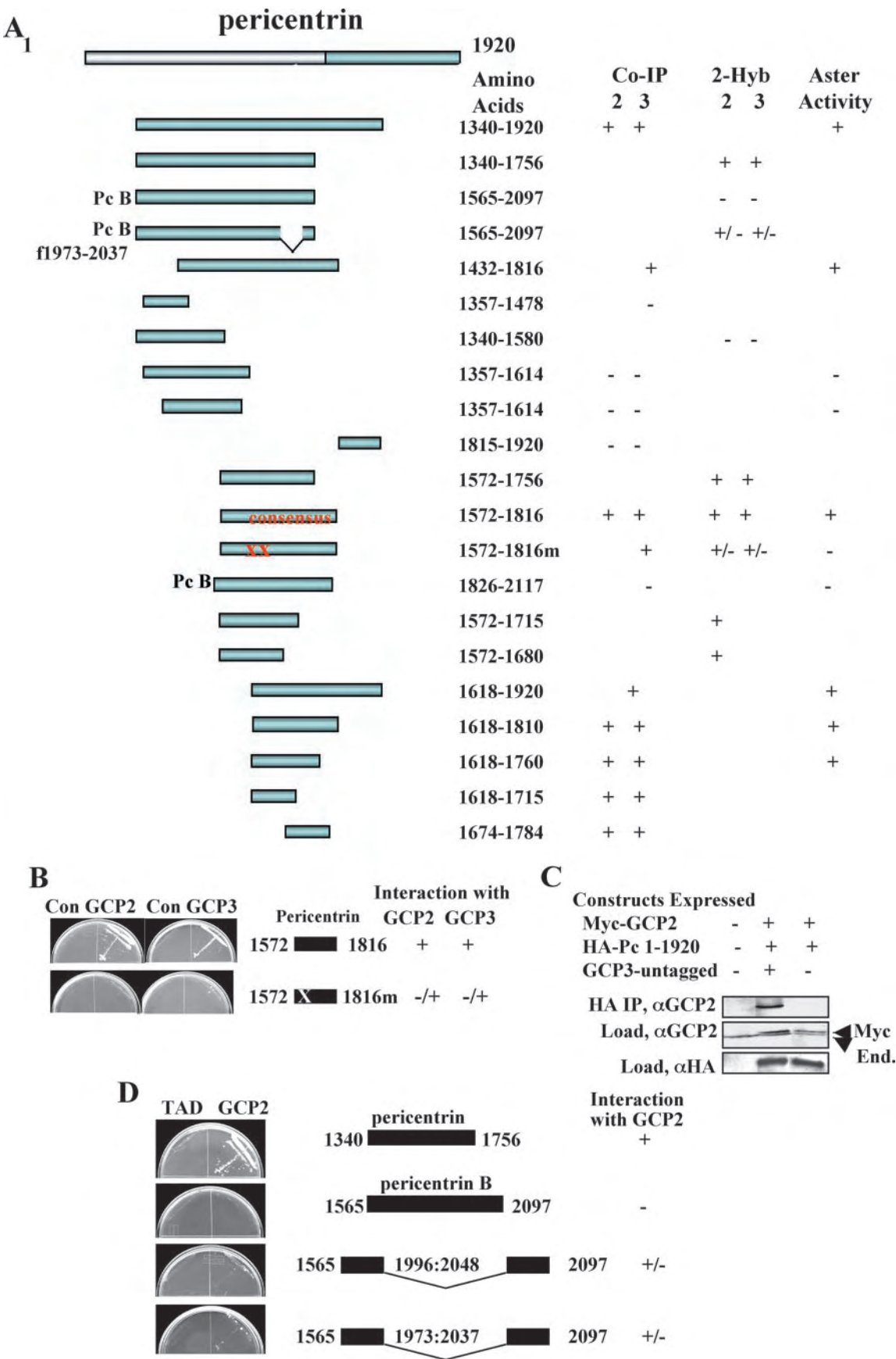
Figure 5.

(20–22 h) and increased to ~90% at later times (44 h post-transfection, Figure 6, H, H', and M). Most monopolar spindles had two duplicated and separated centrosomes. We also observed spindles with reduced numbers of centrosome-associated astral microtubules (Figure 6, C', G, J, and K'), bipolar spindles with shortened pole-to-pole axes (Figure 6, C' and G, minispindles) and half spindles with single focused poles (Figure 6, I–I''). In many spindles, we observed a decrease in the centrosome level of γ tubulin (Figure 6, E and I) and other centrosome proteins (Figure 6C), although the proteins were never reduced to undetectable levels. Pericentrin domains that bound both GCP2 and GCP3 induced the same defects, and those that did not interact had little or no effect (Figure 6, K–K''). Moreover, we were unable to detect aster inhibitory activity in *Xenopus* extracts (Figures 4D and 5A), disruption of spindle organization (Figure 6, M–M') or apoptosis (below) associated with the homologous region of pericentrin B, suggesting that these two molecules may not be functionally analogous. Together, these results suggest that uncoupling of the pericentrin A–γ tubulin interaction in mitotic cells caused a reduction in the centrosome-associated γ TuRCs and disrupted astral microtubules and spindle organization, ultimately producing monopolar spindles.

Overexpression of the GCP2/3 Binding Domain of Pericentrin and Reduction in Pericentrin Levels Both Induce G2/Antephase Delay and Apoptosis

During the course of these studies, we observed a marked reduction in cell density in cultures transfected with the GCP2/3 binding domain (Figure 7, A and A'). Typically, half the cells detached from their substrate by 44 h, whereas there was little change in cell density before protein expression (Figure 7A', 20 h). To investigate this further, we examined cells for apoptosis and found that a significant fraction of the cells stained with an apoptosis-specific marker that detects a caspase 3 product of cytokeratin 18 produced early in apoptosis (Figure 7A'', M30); control cells showed low levels of M30 staining.

Apoptosis required that cells be actively cycling, because we did not detect apoptosis when cells were plated at high density to induce G_1/G_0 arrest during the period of protein expression. In cycling cells of several different origins, we observed a low mitotic index (Figure 7C) suggesting that cells were delayed at some point in the cell cycle. We found that cells accumulated in a premitotic stage based on their ability to stain for a form of histone H3 that is phosphorylated by aurora B in early mitotic cells (Swedlow and Hirano, 2003; Hans and Dimitrov, 2001); control cell staining was significantly lower (Figure 7, D and F). The cell cycle period between late G2 and mitosis (before chromosome condensation occurs) is termed antephase (Pines and Rieder, 2001). Antephase arrest was linked to apoptosis because most early mitotic cells (phospho-H3-positive) were also early apoptotic (Figure 7G, M30-positive). Moreover, most centrosomes in apoptotic cells seemed duplicated and separated (Figure 7H, two γ tubulin spots), consistent with cells in late G2 or early prophase.

To confirm the link between cell cycle arrest and cell death, we microinjected cDNA into nuclei of COS cells arrested in S phase by thymidine block. Approximately 8 h after release from the block cells entered mitosis. At this time, a significant proportion of cells expressing the GCP2/3 binding domain of pericentrin expressed the M30 antigen or detached from the substrate whereas control cells remained attached and often increased in number (Figure 8, A, B, and D). Cell loss was cell cycle specific because premitotic cycling cells or cells kept under S phase arrest remained viable and adherent (Figure 8C). These results suggested that uncoupling the pericentrin–γ TuRC interaction and disruption of astral microtubules induced apoptosis at the G2/M transition. (Figure 8C).

We reasoned that if apoptosis resulted from a cellular defect common to both overexpression and reduction of pericentrin, we should observe cell cycle arrest and apoptosis after pericentrin silencing. Significant cell death was in several cell types knocked down for pericentrin A and B at 48–72 h posttreatment (our unpublished data). Pericentrin A/B silencing also induced a significant increase in antephase, and a decrease in mitotic index 48–72 h after protein silencing (Figure 7, C and D). These provide further support for the idea that antephase arrest and apoptosis may be caused by disruption of the pericentrin–γ tubulin interaction.

Figure 5 (facing page). Summary of GCP2/3 binding and aster inhibitory activity of pericentrin domains. (A) Binding and aster activity of various pericentrin constructs. CoIP, coimmunoprecipitation; 2-hyb, yeast two-hybrid; aster, aster inhibitory activity; consensus, smallest domain identified in both coIP and 2-hyb that has high-affinity binding activity to both GCP2 and GCP3; XX, E to A mutations in 1613 and 1615, Pc B φ 1973–2037 lacks an exon encoding the indicated amino acids. Although variable, interactions of all affinities were scored as + unless they were at the limit of detection (then scored as +/−). No markings such as + or − denote data not acquired for these parameters. Proteins were considered positive in the aster inhibition assay if they showed at least 31% reduction in aster assembly relative to control activity. For clarity, the pericentrin B constructs are arbitrarily sized and aligned with homologous regions of pericentrin. (B) Yeast two-hybrid data showing significantly reduced binding of mutant pericentrin domain for GCP2 and GCP3. (C) Cooverexpression, coimmunoprecipitation data showing enhanced binding of GCP2 to pericentrin 1–1920 in the presence of GCP3 (see Figure 2 legend for details). Con, control. (D) Yeast two-hybrid data showing binding of GCP2 by a pericentrin A fragment and lack of GCP2 binding by the homologous pericentrin B fragment as well as mutants lacking a pericentrin B specific exon (see MATERIALS AND METHODS for details).
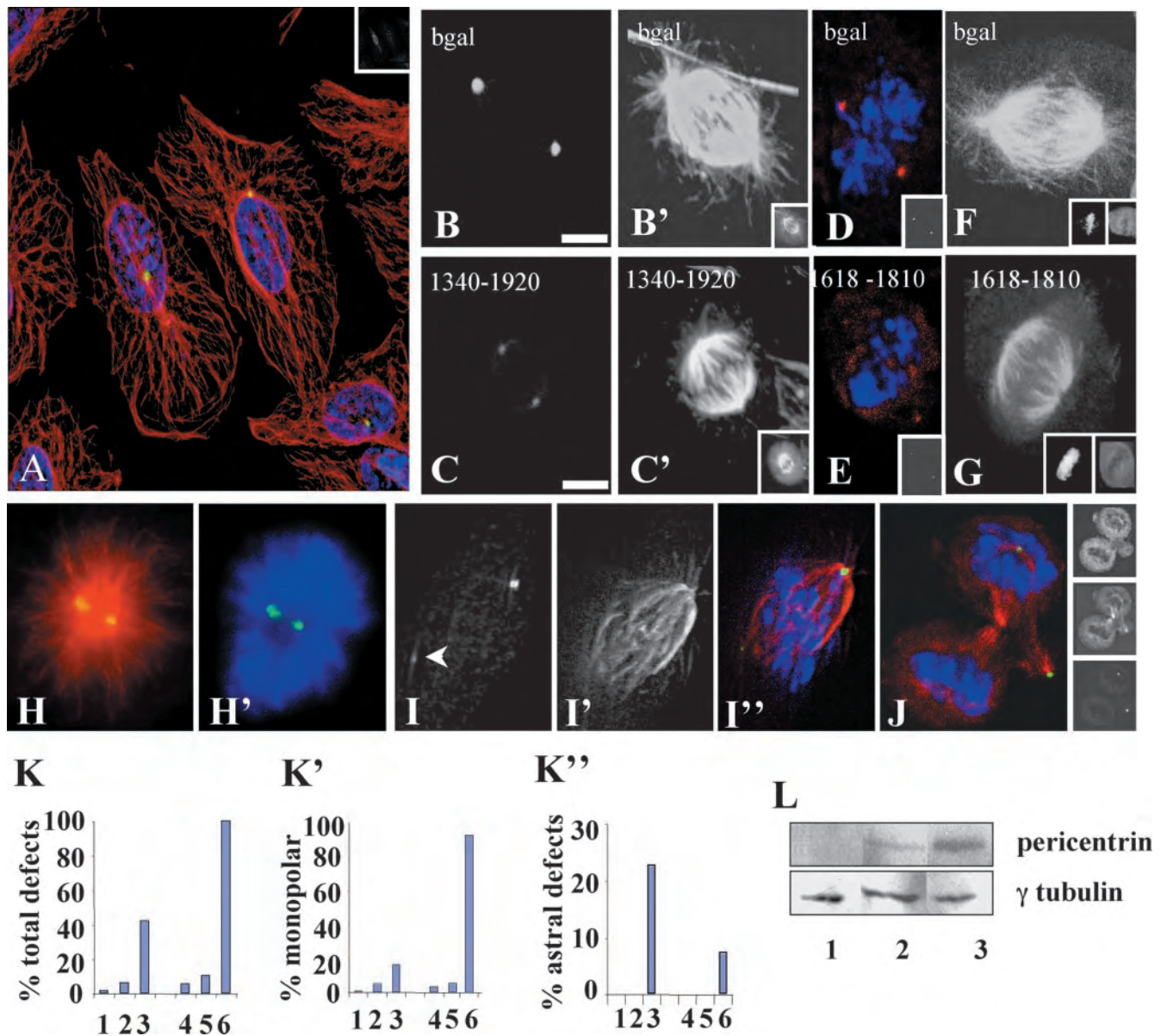
DISCUSSION

Our previous results demonstrated that pericentrin and γ tubulin interacted in *Xenopus* extracts and that the proteins were in proximity at centrosomes in vertebrate cells, suggesting that they interacted at this site as well (Dictenberg *et al.*, 1998). The additional data provided in this study show that pericentrin interacts with the γ TuRC via domains that bind GCP2 and GCP3 and that this interaction is important for microtubule organization in mitotic cells. The results of this study are consistent with our previous work showing that pericentrin overexpression induces severe spindle defects (Purohit *et al.*, 1999). We propose a model in which pericentrin acts as a scaffold for anchoring γ TuRCs at mitotic centrosomes/spindle poles. This interaction seems to be required not only for astral microtubule organization but also for maintaining spindle bipolarity and for mitotic entry. The monopolar spindles and "minispindles" induced by disruption of the pericentrin-GCP2/3 interaction, indicate that pericentrin anchoring of γ TuRCs also may play a role in organizing microtubules of the central spindle.

Figure 6. GCP2/3 binding domain of pericentrin affects astral microtubules and spindle organization in vertebrate cells. (A) Interphase cell expressing pericentrin 1680–1810 (inset, top right) shows no difference in microtubule organization compared with surrounding control cells (red, microtubules; blue, DNA stained with DAPI; yellow, 5051 centrosome staining). (B and B') Control mitotic cell expressing β-galactosidase. (C and C') Cell expressing pericentrin 1340–1920 and spindles with reduced astrals and pole-to-pole distance (C', compare with B and B'). Insets at bottom right of B', C' show protein expression. (D) β-Galactosidase–expressing control cell. (E) Cell expressing pericentrin 1680–1810 shows reduced γ tubulin at spindle poles (compare with D). D and E, γ tubulin (red), DNA (blue), insets show 5051 staining. (F) β-Galactosidase–expressing cell. (G) Cell expressing 1680–1810 (inset, bottom right) shows reduced astral microtubules and decreased pole-to-pole distance compared with F. DNA, insets, left. Overexpressed protein, insets, right. (H) Monopolar spindle in cell expressing 1680–1810 showing centrosomes (yellow, 5051) and microtubules (H) or DNA (H'). (I) Spindle from cell expressing 1680–1810 with one tiny spindle pole (arrowhead, 5051) and unfocused microtubules at this pole (I', merge, I''). (J) Telophase cell expressing 1680–1810 undergoing tripolar division. One nascent daughter cell lacks a centrosome (bottom). Images at right show protein expression (top), microtubules (middle) and centrosomes (bottom). (A–J) Immunofluorescence images of SAOS cells. All images except H and H' are shown with deconvolution. Paired images were stained in parallel and collected on the same day, without modification to the laser or acquisition settings between images. (K–K'') Graphs showing percentage of transfected mitotic SAOS cells with total mitotic defects (K), monopolar spindles (K'), and reduced or absent astral microtubules (K'') 1–3, 20 h posttransfection; 4–6, 40 h posttransfection. 1, nontransfected mitotics, n = 368; 2, βgal, n = 82; 3, 1618–1810, n = 31; 4, Peri B 1826–2117; n = 95. 5, βgal, n = 39. 6; 1618–1810, n = 14. p values comparing βgal- and 1618–1810–expressing cells were calculated using the Student's *t* test for both time points. K, p < 0.0001 at 22 h; p < 0.0001 at 44 h. K', p = 0.049 at 22 h; p < 0.0001 at 44 h. (L) Soluble pericentrin is more abundant in mitotic cells. Western blot of HeLa whole cell lysates from asynchronous cells (lane 1), from cells treated 4 h with nocodazole (lane 2), and from mitotic cells after shake-off (lane 3). Total protein loads were normalized for each lane using the Bio-Rad protein assay.

Centrosomal Anchoring of γ TuRCs by Pericentrin Is Required for Mitotic Microtubule Aster Organization in Xenopus Extracts and Somatic Cells

Our results indicate that pericentrin anchoring of γ TuRCs at centrosomes is required for mitotic aster organization. If anchoring is disrupted, γ tubulin is dramatically depleted at mitotic centrosomes in *Xenopus* extracts and reduced at spindle poles in somatic cells. The more dramatic loss of centrosomal γ tubulin from *Xenopus* asters suggests that pericentrin plays a more dominant role in the organization of γ TuRCs at centrosomes in this system and perhaps in embryonic systems in general. We have not investigated the fate of γ TuRCs once dissociated from centrosomes, although one possibility is that they remain attached to the minus ends of microtubules where they could cap microtubule growth (Wiese and Zheng, 2000). In somatic cells, a fraction of γ tubulin remains at centrosomes/spindle poles under conditions that disrupt the GCP2/3-pericentrin interaction. This fraction could be anchored by other proteins that have been shown to bind γ TuRC components such as AKAP450, pericentrin B (Takahashi *et al.*, 2002) Nlp (Casenghi *et al.*, 2003), and centrosomin (Terada *et al.*, 2003)

In this study, we map the GCP2/3 binding site of pericentrin to the C terminus of the protein, a region that shows no apparent homology to AKAP450, Spc110, Spc72, or CP309 (Kawaguchi and Zheng, 2003; Takahashi *et al.*, 2002), although it is conserved between mouse, human, and rat (66–75% identical, 78–84% similarity). Whereas the amino terminus of pericentrin B binds GCP2 (Takahashi *et al.*, 2002) (W. Zimmerman and S. Doxsey, unpublished observations), a similar region in the smaller pericentrin isoform does not, perhaps because it lacks exons found in pericentrin B. More information on the GCP2/3 interacting domain will require mapping these sites in all the GCP2 binding proteins.

The phenotype observed with the GCP2/3-pericentrin disrupting polypeptides and after pericentrin silencing is similar in many respects to that seen after functional abrogation of γ tubulin and other proteins of the γ TuRC. Under these conditions, centrosomes in *Caenorhabditis elegans* and *Drosophila* embryos were compromised in their ability to form mitotic asters (Hannak *et al.*, 2002; Strome *et al.*, 2001), separate from one another (Barbosa *et al.*, 2003; Sampaio *et al.*, 2001), and organize meiotic and mitotic spindles (Sunkel *et al.*, 1995; Barbosa *et al.*, 2000, 2003). It is of interest that mitotic asters in some of these systems formed in the absence of γ tubulin or other γ tubulin ring complex proteins (Strome *et al.*, 2001; Hannak *et al.*, 2002; Barbosa *et al.*, 2003). This is in contrast to our results in *Xenopus* extracts where microtubule asters did not form in the presence of the pericentrin interacting domain of GCP2/3 even after extended periods (30 min). Moreover, preformed mitotic asters were rapidly disassembled after addition of this polypeptide. Future studies will be required to determine whether pericentrin and γ tubulin are more critical for mitotic aster formation in *Xenopus* extracts than in the other systems, or whether uncoupling γ tubulin from pericentrin prevents both γ tubulin-mediated microtubule nucleation and nucleation by a proposed γ tubulin-independent pathway (Hannak *et al.*, 2002).

Pericentrin Is Not Essential for Assembly and Anchoring of γ TuRCs at Interphase Centrosomes

The GCP2/3-interacting pericentrin domains described in this study had no detectable effect on assembly of asters in interphase extracts prepared from *Xenopus* or in interphase somatic cells. Moreover, silencing of both isoforms also had no apparent effect on localization of γ tubulin at the centrosome or microtubule organization in interphase cells. This suggests that the protein does not play a major role in γ tubulin assembly or anchoring at interphase centrosomes but rather that the aster-organizing function of pericentrin is mitosis specific. Because both proteins are normally present at the centrosome throughout the cell cycle, we cannot conclude that they do not interact during interphase. Only that this specific interaction is not necessary for γ tubulin localization. It has been shown that γ tubulin and associated proteins are crucial for microtubule nucleation from interphase centrosomes (Joshi *et al.*, 1992; Hannak *et al.*, 2002). It is thus likely that proteins other than pericentrin provide microtubule-anchoring sites at centrosomes in interphase cells.

Other Proteins Involved in Centrosomal γ TuRC Anchoring and Microtubule Organization

Several other proteins play a role in centrosome organization and microtubule nucleation. However, their ability to directly anchor components of the γ TuRC and thus serve as molecular scaffolds for tethering these complexes to centrosomes has not been demonstrated. These include the centrosome proteins Asp (do Carmo Avides and Glover, 1999), NuMA (Merdes *et al.*, 1996), TPX-2 (Wittmann *et al.*, 2000; Garrett *et al.*, 2002), SPD-5 (Hamill *et al.*, 2002), PCM-1 (Dammermann and Merdes, 2002), Sas-4 (Kirkham *et al.*, 2003) centrosomin (Megraw *et al.*, 1999; Terada *et al.*, 2003), and several regulatory molecules, including Aurora A (Hannak *et al.*, 2001; Giet *et al.*, 2002), Polo (Lane and Nigg, 1996; Barbosa *et al.*, 2000), PP1 (Katayama *et al.*, 2001), and PP4 (Sumiyoshi *et al.*, 2002).

Some of these proteins play a critical role in a centrosome-independent spindle assembly pathway mediated by the Ran GTPase (see Dasso, 2002) including NuMA (Nachury *et al.*, 2001; Wiese *et al.*, 2001) and TPX-2 (Gruss *et al.*, 2001). This is in contrast with pericentrin, which seems to be critical for assembly of mitotic asters but not Ran-mediated asters. In this regard, the proposed function of pericentrin in aster formation also differs from that of epsilon tubulin, which seems to be required for centrosome-independent but not centrosome-dependent microtubule aster formation (Chang *et al.*, 2003). From this discussion, it seems that different molecules are required to organize asters in centrosome-dependent and -independent pathways as well as at different stages of the cell cycle.

Regulation of the Pericentrin–GCP2/3 Interaction

Pericentrin, γ tubulin, and γ tubulin-associated proteins are localized to centrosomes throughout the cell cycle (Stearns *et al.*, 1991; Zheng *et al.*, 1991; Dictenberg *et al.*, 1998). However, the pericentrin–GCP2/3 interaction seems to be involved in γ TuRC anchoring only during mitosis. This suggests that the interaction of pericentrin and γ TuRCs is regulated. The mechanism and regulation of cell cycle-specific binding between these centrosome components is unknown. One model is that γ TuRCs are anchored to different centrosome scaffold proteins at different cell cycle stages and that these interactions are regulated in a cell cycle-dependent manner. For example, the γ TuRC binding activity of pericentrin could be regulated by phosphorylation by mitotic kinases. γ TuRC binding also could be regulated at least in part, through differential patterns of protein expression. Consistent with this idea is the observation that pericentrin, which is expressed primarily in mitosis and in tissues that are highly proliferative (Doxsey *et al.*, 1994; Figure 6N), has a mitotic phenotype. Future experiments will be required to determine the contribution of these and other centrosome
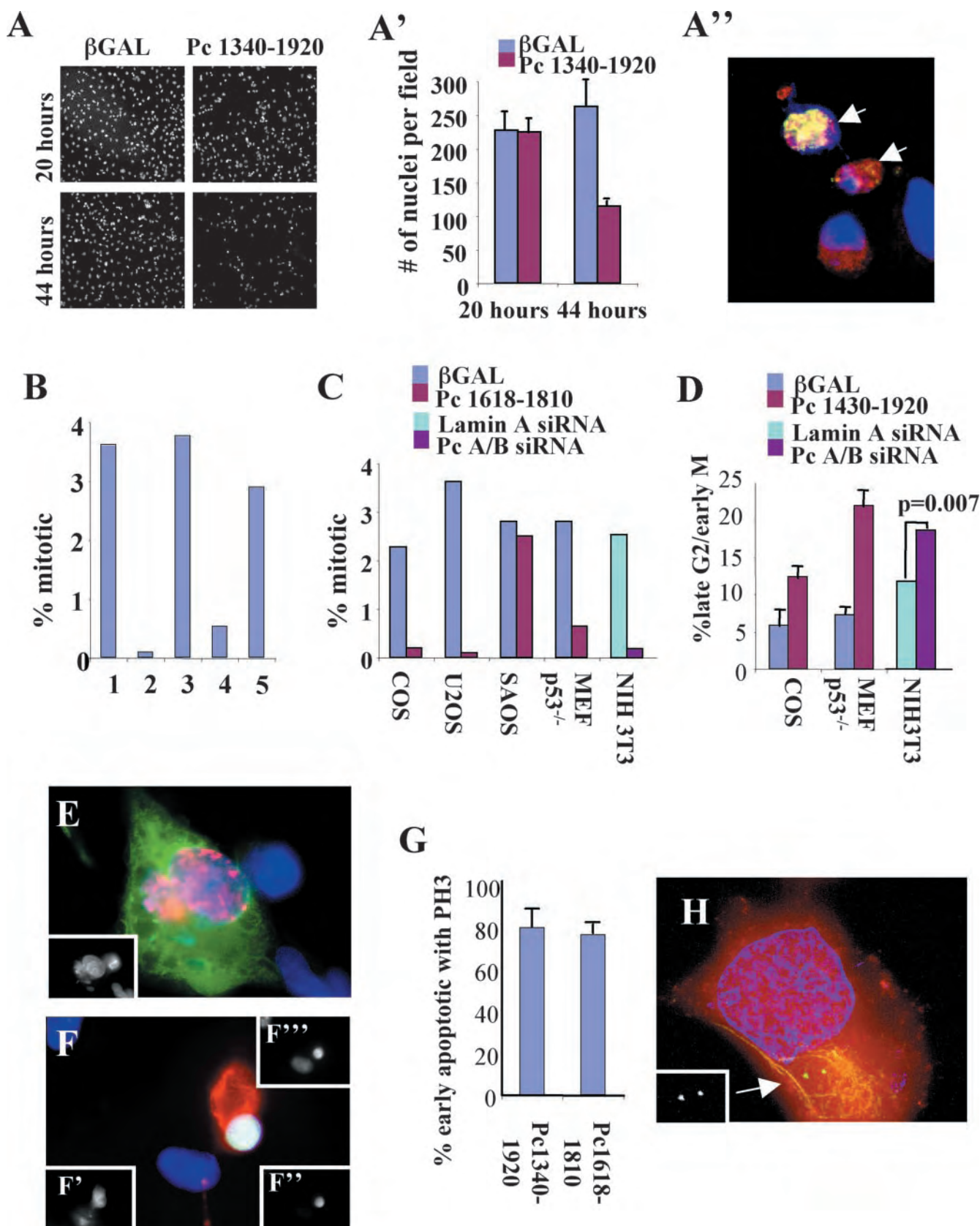
Figure 7. Overexpression of GCP2/3 binding domain or silencing of Pc A/B induces cell cycle arrest and apoptosis at the G2/M phase of the cell cycle. (A) Cells expressing the GCP2/3 binding domain are lost through apoptosis. Low-magnification image of COS cells stained with DAPI showing cell loss when 1340–1920 is expressed for 44 h compared with nonexpressing cells (20 h) or β-galactosidase (βgal)–expressing cells. (A′)

proteins in the anchoring of γ TuRCs to centrosomes at different cell cycle stages.

G2/Antephase Delay and Apoptosis

G2 accumulation of cells expressing the GCP2/3 binding domain of pericentrin or after silencing of pericentrin A/B suggests that disruption of the pericentrin γ TuRC interaction in vivo elicits a checkpoint response at this time in the cell cycle. Recent studies have implicated γ tubulin as well as the Spc110p homologue Pcp1p in regulation of the metaphase to anaphase transition (Prigozhina *et al.*, 2004; Rajagopalan *et al.*, 2004), but this is the first study suggesting a role for these or related molecules in regulation of mitotic entry. We do not yet know what this checkpoint may be monitoring. We favor a model in which the checkpoint senses spindle pole assembly/centrosome maturation because disruption of the γ tubulin–pericentrin interaction disrupts spindle pole assembly and possibly centrosome maturation, which increases in size fourfold between G2 and early prophase (Piehl *et al.*, 2004), concurrent with the onset of γ tubulin mislocalization and antephase arrest that we observe.

Our results showing that pericentrin A/B silencing has no significant affect on interphase microtubule arrays confirms previous work (Dammermann and Merdes, 2002). In this earlier study, the authors did not address mitotic defects most likely because a G2 checkpoint is activated, apoptosis follows, and mitotic cells are rarely observed, a phenomenon that we have encountered in two of the cell lines used by these authors; U2OS and HeLa (Figure 7C; data not shown). In this study, we overcame this problem by using a cell line that that apparently lacks this checkpoint and fails to undergo apoptosis.

Apoptosis is commonly observed after checkpoint activation if a cellular imbalance cannot be repaired. We have not determined which molecular pathway is involved in the G2/antephase arrest identified in this study. DNA damage induces two molecularly distinct pathways involved in G2 arrest, one ATM dependent, the other ATM independent (Xu *et al.*, 2002). Cellular insults other than DNA damage

Figure 8. COS cells expressing Pc1618–1810 undergo apoptosis during the G2/M transition. (A) Cells expressing 1618–1810 undergo apoptosis at mitosis. Microinjected cells released from a double thymidine block were stained for DNA (DAPI) and overexpressed protein (250 cells/bar). 1618–1810–expressing cells were apoptotic or detached 8–10 h after injection, whereas control cells (Pc B1826–2117) increased in number. At 14 h, p value comparing the two treatments, p < 0.0001 (B) Mitotic index of COS cells after release from double thymidine block as in A (peak, 9 h). (C) 1618–1810–expressing cells arrested in S phase do not undergo apoptosis. Microinjected cells retained in thymidine for the times indicated. No loss of cells was observed 8–14 h later. 10*, microinjected cells arrested in S phase for 6 h and then released from the block for 4 h (10 h total, p = 0.25). (D) Immunofluorescence image of an apoptotic cell expressing 1618–1810 at 10 h postinjection as in A. D (overlay), DNA (blue and upper inset), M30 (red), overexpressed protein (lower inset).

Figure 7 (cont). Quantification of cells in A (mean and SD, 10 fields). (A″) Image of COS cells from A stained for DNA (blue), 1340–1920 (red) and M30 cytodeath (green). Arrows, cells with both 1340–1920 and M30. (B) Mitotic index of U2OS cells expressing: 1, βgal; 2, 1618–1810 p < 0.001; 3, Pc B1826–2117 p = 0.437; 5, 1572–1816 p = 0.011; 6, 1572–1816m p = 0.226 (n = 1000 cells/bar at 40–44 h posttransfection). p values were calculated using the *t*-test relative to βgal controls. (C) Mitotic index in indicated cell types overexpression the indicated constructs or treated with siRNA. p values calculated as described above: COS, p < 0.001; U2OS, p < 0.001; SAOS, p = 0.479; Mefp53−/−, p = 0.001; NIH3T3 (siRNA), p = 0.0004. (D) Cells expressing GCP2/3 binding domain constructs or treated with Pc A/B siRNA have a greater proportion of late G2 cells. Shown are mean and SD of three experiments or p value based upon scoring from 1000 treated cells. Cells immunostained for overexpressed protein (green), phosphorylate histone H3 (red), and DAPI (blue). (E) Antephase cell overexpressing 1340–1920 (stains for phosphorylated histone H3 and does not show condensed chromatin). Inset, DAPI. (F) Apoptotic antephase cells expressing 1340–1920. M30 (red), phosphorylated histone H3 (green; inset, bottom right), DNA (blue; inset, above), overexpressed protein (inset, bottom left). (G) Graph showing that most early apoptotic cells expressing GCP2/3 binding domains stain for phosphorylated histone H3. Shown are mean, SD, n = 3 experiments. (H) Early apoptotic cell expressing 1340–1920 stained for centrosomes (5051, green), DNA (blue), HA pericentrin (red), M30, yellow. Inset 5051. Imaged as in Figure 5.

also can induce late G2 arrest, including microtubule disruption, hypothermia, fluoride treatment, and viral protein expression (Pines and Rieder, 2001; Tyler *et al.*, 2001; Elder *et al.*, 2002; Mikhailov and Rieder, 2002).

Antephase delay and subsequent apoptosis also can be activated through pathways that include p53 and Rb. Our data demonstrate that that p53 is not involved because primary MEFs lacking p53 retain the checkpoint response. SAOS cells, which do not arrest and apoptose have been reported to lack Rb (Scolnick and Halazonetis, 2000). We are currently testing the role of Rb in the checkpoint response. We also are investigating other mechanisms for inducing apoptosis. For example, apoptosis can be triggered by mislocalization of antiapoptotic signals from centrosomes (Li, 1998). γ Tubulin has recently been shown to associate with DAP-like kinase, which is implicated in apoptosis (Preuss *et al.*, 2003). However, the role of γ tubulin mislocalization from centrosomes and induction of apoptosis through DAP-like kinase has not been explored. Mislocalization of survivin and other antiapoptotic proteins from centrosomes can induce apoptosis (Reed and Reed, 1999; Piekorz *et al.*, 2002; Sandal *et al.*, 2003). Additional studies will be required to identify the proteins and pathways involved in the apoptotic response observed after disruption of the pericentrin–GCP2/3 interaction.

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Mitosis-specific Anchoring of γ Tubulin Complexes by Pericentrin Controls Spindle Organization and Mitotic Entry

Wendy C. Zimmerman, James Sillibourne, Jack Rosa, and Stephen J. Doxsey*

Department of Molecular Medicine, University of Massachusetts Medical School, Worcester, Massachusetts 01605

Microtubule nucleation is the best known function of centrosomes. Centrosomal microtubule nucleation is mediated primarily by γ tubulin ring complexes (γ TuRCs). However, little is known about the molecules that anchor these complexes to centrosomes. In this study, we show that the centrosomal coiled-coil protein pericentrin anchors γ TuRCs at spindle poles through an interaction with γ tubulin complex proteins 2 and 3 (GCP2/3). Pericentrin silencing by small interfering RNAs in somatic cells disrupted γ tubulin localization and spindle organization in mitosis but had no effect on γ tubulin localization or microtubule organization in interphase cells. Similarly, overexpression of the GCP2/3 binding domain of pericentrin disrupted the endogenous pericentrin–γ TuRC interaction and perturbed astral microtubules and spindle bipolarity. When added to *Xenopus* mitotic extracts, this domain uncoupled γ TuRCs from centrosomes, inhibited microtubule aster assembly, and induced rapid disassembly of preassembled asters. All phenotypes were significantly reduced in a pericentrin mutant with diminished GCP2/3 binding and were specific for mitotic centrosomal asters as we observed little effect on interphase asters or on asters assembled by the Ran-mediated centrosome-independent pathway. Additionally, pericentrin silencing or overexpression induced G2/antephase arrest followed by apoptosis in many but not all cell types. We conclude that pericentrin anchoring of γ tubulin complexes at centrosomes in mitotic cells is required for proper spindle organization and that loss of this anchoring mechanism elicits a checkpoint response that prevents mitotic entry and triggers apoptotic cell death.

INTRODUCTION

The centrosome is the primary microtubule-organizing center in animal cells. At the centrosome core is a pair of barrel-shaped microtubule assemblies, the centrioles (Doxsey, 2001). Centrioles are capable of self-assembly (Marshall *et al.*, 2001; Khodjakov *et al.*, 2002) and can serve as templates for recruitment and organization of the surrounding pericentriolar matrix (Bobinnec *et al.*, 1998; Kirkham *et al.*, 2003). The pericentriolar material or centrosome matrix contains a high proportion of coiled coil proteins and is the site of microtubule nucleation. Within the matrix are large protein complexes of γ tubulin and associated proteins that have a ring-like structure and mediate the nucleation of microtubules called γ tubulin ring complexes or γ TuRCs (Moritz *et al.*, 1995a; Zheng *et al.*, 1995). Other proteins may share the ability to nucleate microtubules because centrosomes can organize microtubules in the absence of functional γ tubulin (Sampaio *et al.*, 2001; Strome *et al.*, 2001; Hannak *et al.*, 2002).

During cell cycle progression, centrosomes "mature" by recruiting additional γ TuRCs and several other proteins, resulting in an increase in the nucleation capacity of the centrosome (reviewed in Blagden and Glover, 2003). How-

ever, we still know very little about proteins that directly anchor γ TuRCs to centrosomes in vertebrate cells. In the budding yeast, a small γ tubulin complex composed of γ tubulin (Tub4p), Spc97p, and Spc98p (~700 kDa) is bound to the nuclear side of the spindle pole body (the centrosome equivalent) through an interaction with Spc110p (Knop and Schiebel, 1997) and to the cytoplasmic side of the spindle pole body through Spc72p (Knop and Schiebel, 1998). Spc97p and Spc98p mediate binding of the complex to Spc110p and Spc72p (Knop and Schiebel, 1997; Knop and Schiebel, 1998; Nguyen *et al.*, 1998). Although there is no apparent homology between their SPC97/98 interacting domains, chimeras formed by fusing the binding domain of one with the localization domain of the other can rescue knockouts of the proteins encoding the localization domains, suggesting that the two binding domains are functionally homologous (Knop and Schiebel, 1998).

γ TuRCs in vertebrate cells and *Drosophila* contain orthologues of the three yeast proteins (γ tubulin and γ complex proteins 2 and 3 [GCP2, 3]) as well as several additional components (Zheng *et al.*, 1995; Martin *et al.*, 1998; Moritz *et al.*, 1998; Murphy *et al.*, 1998, 2001; Oegema *et al.*, 1999; reviewed in Job *et al.*, 2003). In vertebrates, the centrosome protein pericentrin (pericentrin A) forms a large complex with γ tubulin in the cytoplasm, and the two proteins are also in proximity at the centrosome (Dictenberg *et al.*, 1998). Recent evidence suggests there may be as many as 10 isoforms of pericentrin in human cells (Flory and Davis, 2003). A large isoform (pericentrin B/kendrin; Flory and Davis,

2003) and another centrosome protein called AKAP450/GC-NAP share homology with the calmodulin binding domain of Spc110p (Flory *et al.*, 2000; Gillingham and Munro, 2000; Li *et al.*, 2001). Other potential Spc110p orthologues have been identified in *Schizosacharomyces pombe*, *Aspergillus nudulans*, and *Drosophila* based on sequence homology (Flory *et al.*, 2002; Kawaguchi and Zheng, 2003) and in vertebrates (*Xenopus* and human) based on immunological cross-reactivity with Spc110p-specific antibodies (Tassin *et al.*, 1997). All proposed vertebrate orthologues of Spc110p localize to the centrosome and coimmunoprecipitate with γ TuRCs (Tassin *et al.*, 1997; Dictenberg *et al.*, 1998; Takahashi *et al.*, 2002). No Spc72p orthologues have been identified in other species.

In vertebrate cells, pericentrin B and AKAP450 have recently been shown to bind GCP2 in vitro (Takahashi *et al.*, 2002). Antibody inhibition and immunodepletion studies demonstrated a role for pericentrin isoforms and AKAP450 in microtubule nucleation in vertebrates and *Drosophila* (Doxsey *et al.*, 1994; Takahashi *et al.*, 2002; Kawaguchi and Zheng, 2003; Keryer *et al.*, 2003), perhaps by localizing the small Ran GTPase to centrosomes (AKAP450) (Keryer *et al.*, 2003). However, other studies show that antibody depletion of pericentrin B or reduction of pericentrin A and B do not affect aster formation, microtubule organization, or centrosome-associated γ tubulin (Li *et al.*, 2001; Takahashi *et al.*, 2002; Dammermann and Merdes, 2002). Moreover, loss of AKAP450 from centrosomes does not affect centrosomal γ tubulin localization, even though microtubule organization is disrupted (Keryer *et al.*, 2003). Another potential centrosomal γ TuRC-anchoring protein has recently been identified in vertebrate cells called ninein-like protein (Nlp), which can bind γ TuRC complexes, inhibit nucleation when neutralized with antibodies, and enhance nucleation when overexpressed (Casenghi *et al.*, 2003). However, we know little about the role of these putative scaffold proteins in centrosomal anchoring of γ TuRCs during the cell cycle and the cellular consequences of specifically disrupting their interactions with γ TuRCs at centrosomes.

In this study, we show that siRNAs targeting both pericentrin isoforms (A and B) induced specific loss of γ tubulin from spindle poles in mitosis, reduction of astral microtubules, and formation of monopolar spindles. This phenotype seemed to be specific for the smaller isoform of pericentrin because it was not observed when the larger pericentrin isoform was specifically reduced. A region at the C terminus of pericentrin interacted with both GCP2 and GCP3 in vitro as shown by coimmunoprecipitation and two-hybrid analysis. Expression of the GCP2/3 binding domain of pericentrin produced a phenotype similar to that observed in cells with reduced pericentrin. It disrupted the interaction between endogenous pericentrin and γ TuRCs, and adsorbed γ TuRCs from cell extracts. It reduced astral microtubules and centrosomal γ tubulin in mitotic cells and induced formation of small spindles and monopolar spindles. No effect on interphase microtubules was observed. When added to *Xenopus* extracts this domain dissociated γ tubulin from mitotic centrosomes and rapidly induced mitotic aster disassembly. The loss of γ tubulin from centrosomes in cells with reduced pericentrin levels or in cells expressing the GCP2/3 binding domain of pericentrin ultimately triggered a checkpoint inducing G2/antephase arrest and apoptosis in somatic cells. These phenotypes were not observed after specific reduction in the levels of the larger pericentrin isoform, expression of a mutant pericentrin defective in GCP2/3 binding, or expression of a homologous region of pericentrin B. We conclude that the smaller isoform of pericentrin provides a molecular scaffold for centrosomal anchoring γ TuRCs during mitosis in both embryonic and somatic cell systems.

MATERIALS AND METHODS

Molecular Cloning

All pericentrin constructs used in this study were cloned into pcDNA vectors (Invitrogen, Carlsbad, CA) with amino terminal hemagglutinin (HA) tags (Purohit *et al.*, 1999; Purohit *et al.*, 2001), except those used in two-hybrid studies (see below). Fragments of pericentrin and other genes were polymerase chain reaction (PCR) amplified from cDNAs by using primers with *Not*I and *Xba*I restriction sites. PCR products were digested with the appropriate enzymes, cloned into the vector, and sequences were confirmed. In some cases, EcoR1 and *Xho*I restriction sites were used (peri B1826-2117, 1572-1816, 1572-1816m). GCP2-, GCP3-, and γ tubulin-containing constructs were obtained from Dr. Tim Stearns (Stanford University, Stanford, CA).

Small Interfering RNA (siRNA)

Twenty-one nucleotide RNAs were chemically synthesized by Dharmacon Reasearch (Lafayette, CO). and introduced to cells using Oligofectamine (Invitrogen, Carlsbad, CA.) in accordance with the manufacturer's instructions. The target sequences used were AAUUGGAACAGCUGCAGCAGA against pericentrin A and B in human (Dammermann and Merdes, 2002), AAUGAGGUUGUCCACAGGAGA against pericentrin A and B in mouse, and AAGCUCUGAUUUAUCAAAAGA against the PACT domain of pericentrin B in human. AACUGGACUUCCAGAAGAACA, which targets human lamin A and is nonspecific in mouse, was used as a control for all siRNA studies. Crude cell lysates were analyzed for protein silencing. Cells were treated with 2 mM thymidine for 18 h starting 24 h post-siRNA treatment. Six hours after thymidine release, cells were harvested and lysed in phosphate-buffered saline (PBS) supplemented with 1% Triton X-100, 10 μg/ml leupeptin, 10 μg/ml pepstatin, 10 μg/ml chymotripsin,10 μg/ml phenylmethylsulfonyl fluoride, 2.0 μg/ml *p*-amino-benzamidine, 5 mM iodoacetamide, and 5 mg/ml *N*-ethylmaleimide. Cell lysates were clarified at top speed in a Microfuge for 15 min at 5°C. Protein concentration for each lysate was determined using Bio-Rad protein dye reagent, loads were adjusted, proteins were resolved by SDS-PAGE, and analyzed by Western Blot.

Antibodies

Anti-myc, anti-γ-tubulin, and anti-tubulin antibodies were obtained from Sigma-Aldrich (St. Louis, MO). Phosphohistone H3 rabbit polyclonal antibody was purchased from Upstate Biotechnology (Lake placid, NY). M30 Cytodeath and anti-HA rat monoclonal antibody 3F10 was obtained from Roche Diagnostics (Indianapolis, IN) Anti-human lamin A/C antibody was purchased from Cell Signaling Technology (Beverley, MA). Other antibodies included M8 anti-pericentrin antibody, (Dictenberg *et al.*, 1998), human autoimmune serum 5051 that recognizes centrosome proteins (Doxsey *et al.*, 1994), anti-pericentrin B/kendrin-specific antibody (Flory et.al., 2000) (obtained from Trisha Davis, University of Washington, Seattle, WA), anti-GCP2 antibody (Murphy *et al.*, 1998) (obtained from Dr. Tim Stearns), and anti-GCP3 antibody (a gift from Michel Bornens, Institut Curie, Paris, France).

Yeast Two-Hybrid Cloning/Methods

Direct yeast two-hybrid interactions were performed essentially as described previously (Gromley *et al.*, 2003). Pericentrin, γ tubulin, GCP2, and GCP3 coding sequences were amplified from plasmid DNA by PCR by using Pfu Turbo (Stratagene, La Jolla, CA), cloned into either pGBKT7 or pGADT7 (BD Biosciences Clontech, Palo Alto, CA) and completely sequenced. Yeast strains AH109 and Y187 were transformed with GAL4 DNA binding domain (GAL4-DBD) or GAL4 transactivation domain (GAL4-TAD) expression constructs, respectively, and diploid strains generated by mating. Interactions between pericentrin and members of the γ tubulin ring complex were tested for by streaking yeast onto synthetic defined (SD) medium lacking leucine, tryptophan, histidine, and adenine.

Biochemical Techniques

Immunoprecipitations from *Xenopus* extracts were performed as described previously (Dictenberg *et al.*, 1998) by using the antibodies to the pericentrin amino terminus (M8) (Doxsey *et al.*, 1994) and γ tubulin (Zheng *et al.*, 1995). For disruption of γ TuRCs from pericentrin in coimmunoprecipitations, active or heat denatured pericentrin fractions were added directly to *Xenopus* high-speed extracts before immunoprecipitation. Protein affinity experiments to recruit γ TuRCs (Figure 2) were performed using partially purified fractions of pericentrin domains (see below). Proteins were bound to anti-HA beads, added to extracts for 60 min, washed in extract buffer (Murray, 1991), run on SDS gels, and probed with the indicated antibodies.

Proteins for recruitment of γ TuRCs (Figure 2) and for aster inhibition assays (Figures 4 and 5) were produced in COS cells and purified as follows. Confluent COS cells were transiently transfected with 3 μg of DNA/60-mm
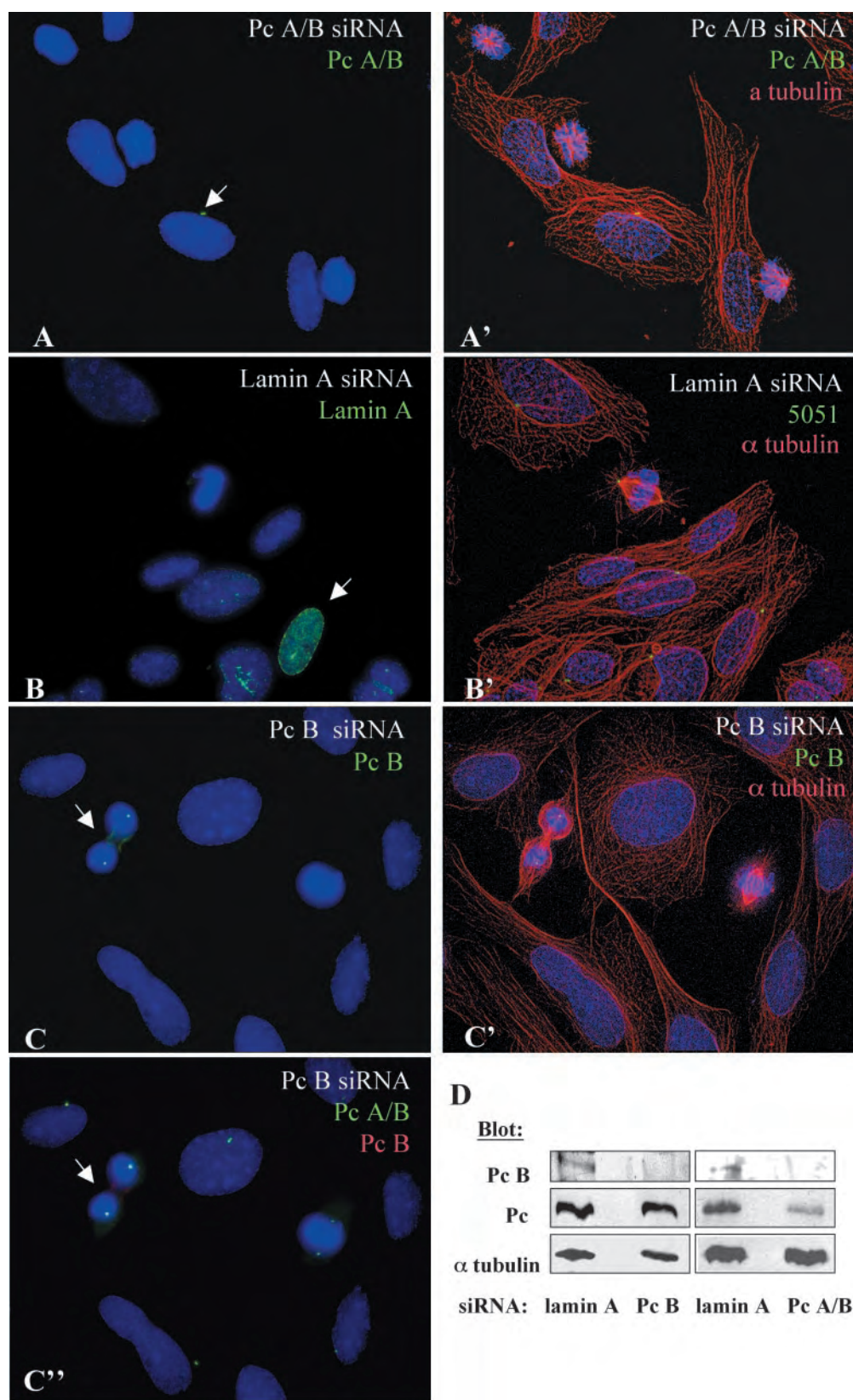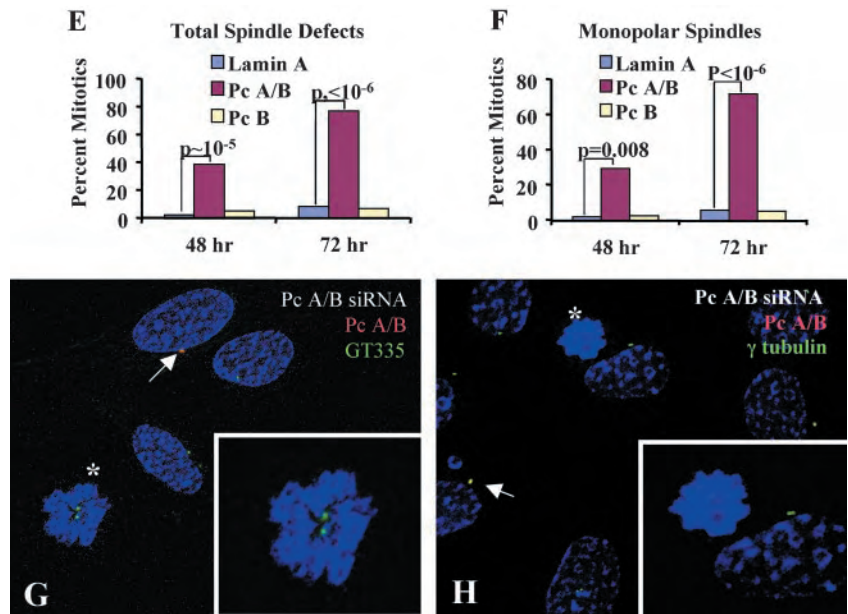
Figure 1.

Figure 1 (cont). Silencing of pericentrin A and B causes mitotic defects. (A) SAOS cells with reduced pericentrin after siRNA treatment stained with a pericentrin antibody (M8, green), which recognizes both isoforms of pericentrin (Pc A/B) and DNA (DAPI, blue). (A') Same field as in A costained for microtubules (α tubulin, red). (B) Cells with reduced lamin A stained for lamin A (green) and DNA. (B') Same field as in B stained for centrosomes with 5051 autoimmune sera (green) and microtubules (red). (C and C') Cells with reduced pericentrin B (targeting the C-terminal PACT domain) stained with pericentrin B-specific antibody, Pc B, (C, green), together with microtubule label (C') or pericentrin antibody that recognizes both isoforms (C''). (A, B, C, and C'') Maximum projection of z series without deconvolution. (A', B', and C') Maximum projection of deconvolved z series. Note the improvement in the resolution of the DNA. Arrows indicate cells expressing near normal levels of the targeted protein. (D) Western blots of crude cell lysates demonstrating reduction of pericentrin B (Pc B) but not pericentrin A (Pc) by using Pc B-specific siRNA or reduction of both isoforms relative to lamin A siRNA control, by siRNA targeting Pc A and Pc B (Pc A/B). Peri-

centrin isoforms probed with Pc A/B anti-pericentrin antibody (M8). α Tubulin loading control probed with DM1α anti-alpha tubulin antibody. (E) Graph showing percentage of mitotic SAOS cells with spindle defects (monopolar, multipolar, and reduced astral microtubules) at 48 and 72 h after siRNA treatment. One hundred to 150 mitotic cells scored per bar. (F) Graph indicating percentage of mitotic cells with monopolar spindles at 48 and 72 h. (G) Cells with reduced pericentrin A/B (Pc A/B, red) retain the centriole marker GT335 (green) in interphase and mitosis. (H) γ Tubulin in cells with reduced pericentrin A/B seems largely unchanged at centrosomes in interphase but is reduced at spindle poles. Pc A/B (red), γ tubulin (GTU88, green), and DAPI (blue). (G–H) Maximum projection of z series with no neighbor deconvolution. Arrows indicate cells with near normal staining levels of pericentrin. Asterisks indicate mitotic cells. Mitotic cells are show at higher magnification in insets.

dish by using LipofectAMINE Plus reagent (Invitrogen). Transfected cells were maintained for 3 d in DMEM with 5% serum and then collected with 5 mM EDTA in PBS. Cells were lysed in PBS supplemented with protease inhibitor cocktail (#1836153; Roche Diagnostics, Basel, Switzerland), 1% Triton X-100, and 5 mg/ml N-ethylmaleimide. For recruitment of γ tubulin from extracts, HA beads were prepared by pretreating Dynabeads 450 (#110.05; Dynal, Lake Success, NY) with a saturating amount of anti-HA antibody 12CA5 (Covance, Denver, PA). Anti-HA IgG beads were treated with COS cell lysate containing an excess of the indicated HA-tagged pericentrin polypeptides, washed three times in PBS lysis buffer, two times in PBS, and two times in extract buffer, before addition of *Xenopus* extracts for γ TuRC recruitment experiments. For preparation of soluble HA-tagged pericentrin, 12CA5 antibody was cross-linked to protein A beads (Bio-Rad, Hercules, CA) by using standard methods (Harlow and Lane, 1988). HA-tagged pericentrin was batch depleted from COS lysates by incubation with HA cross-linked beads at 5°C with gentle agitation for 1 h. Treated beads (configured as a column) were washed with 10 column volumes of lysis buffer, 10 volumes of PBS with protease inhibitors, and 10 volumes of 10 mM Tris, pH 8.0. HA-tagged pericentrin was eluted with 2 volumes of 150 mM glycine, pH 2.5, into 1/4 volume of 1 M Tris, pH 8.0, and dialyzed against PBS overnight.

Coimmunoprecipitations

Coimmunoprecipitation of pericentrin isoforms and γ TuRC components (Figure 3) was performed in COS cells 40–48 h after transient cotransfection of the indicated constructs by using LipofectAMINE Plus reagent. Cells were collected using 5 mM EDTA in PBS. Cell pellets were lysed with 1% NP-40, 1 mM dithiothreitol, 10% glycerol in buffer C (100 mM PIPES, pH 6.9, 6 mM MgCl₂, 0.5 mM EGTA, 10 μg/ml leupeptin, 10 μg/ml pepstatin, 10 μg/ml chymotripsin, 10 μg/ml phenylmethylsulfonyl fluoride, 2.0 μg/ml *p*-aminobenzamidine, 5 mM iodoacetamide). Lysates were clarified 15 min at top speed in a Microfuge at 5°C and then applied to HA Dyna beads (see above). Beads were treated for 1 h at 5°C with end-over-end agitation and washed two times in lysis buffer (see above) and two times in wash buffer (buffer C with 100 mM Na acetate, pH 6.9). Loads and treated beads (immunoprecipitates) were analyzed by SDS gel electrophoresis and Western blot by using the indicated antibodies.

Xenopus Extracts

Cytostatic factor (CSF)-arrested *Xenopus* extracts were prepared, and aster assembly assays were preformed as described previously (Murray, 1991;

Stearns and Kirschner, 1994). For purpose of quantization, two hundred sperm were counted and scored for the presence of assembled microtubules. In some cases, the standard fix [0.3 volume of 37% formaldehyde, 0.6 volumes of 80% (wt/vol) glycerol, 0.1 volume 10× MMR, 1 μg/ml 4,6-diamidino-2-phenylindole (DAPI)] was modified by the addition of 0.05% Oligreen (Molecular Probes, Eugene, OR) to facilitate visualization of sperm nuclei with a scanning confocal microscope. Centrosome assembly in the presence of nocodazole was preformed using published methods (Stearns and Kirschner, 1994). Treated nuclei were prefixed in 5% formaldehyde, spun onto coverslips through a 20% sucrose cushion by using a JS13.1 rotor at 8000 rpm 15 min, and postfixed in methanol (−20°C) before staining for immunofluorescence. Ran-mediated asters were prepared using constitutively active RanL43E as described previously (Wilde and Zheng, 1999). Centrosome-dependent and -independent *Xenopus* mitotic asters were fixed in formaldehyde on coverslips as described previously (Murray, 1991; Wilde and Zheng, 1999).

Cell Lines

Cell lines (COS-7, SAOS, and U2OS) were grown as described previously (American Type Culture Collection, Manassas, VA) and prepared for transfection experiments as described previously (Purohit *et al.*, 1999). Primary mouse embryonic fibroblasts (MEFs) were obtained from Dr. Geoffrey Wahl (Salk Institute for Biological Studies, La Jolla, CA) and used at less than six passages.

Transfection and Immunofluorescence

For transfection and immunofluorescence analysis, logarithmically growing cells were transfected as indicated by the manufacturer with 1 μg of DNA/35-mm dish of the appropriate construct by using LipofectAMINE Plus (Invitrogen) for COS cells and LipofectAMINE for SAOS and U2OS cells. The transfection efficiency for COS cells with control constructs ranged from 35 to 60%. MEFs were transfected using Superfect (QIAGEN, Valencia, CA). For immunofluorescence, cells were fixed with −20°C MeOH as described previously (Purohit *et al.*, 1999). Data were collected as a Z series for deconvolution with 0.3 μm between planes. Images were deconvolved using MetaMorph software, no neighbors algorithm. All images were rendered two dimensional by showing maximum intensity at each point.

Microinjection Experiments

For microinjection, COS cells were synchronized by thymidine block. Cells were treated 16 h with 2 mM thymidine and released (single block), or treated

for an additional 16 h with 2 mM thymidine after 8 h of release (double block). The mitotic index of synchronized cells was determined using replicate coverslips, fixed and stained with DAPI, and then counted at the indicated times postrelease from thymidine block. 1000 cells were counted for each time point. Microinjection into the nucleus of released cells was performed using an Eppendorf transjector 5246, with Eppendorf femtotips, with an injection pressure of 100 hPa, injection time of 0.4 s, and DNA at a concentration of 0.2 μg/μl in PBS.

RESULTS

Pericentrin Silencing Mislocalizes γ Tubulin from Spindle Poles and Disrupts Spindle Bipolarity

We previous showed that pericentrin interacts with the γ tubulin ring complex and that pericentrin antibodies disrupt spindle organization and function (Doxsey *et al.*, 1994; Dictenberg *et al.*, 1998). In this study, we address the molecular mechanism of the mitotic function of pericentrin. Initially, we used siRNAs designed to silence the two previously characterized isoforms of pericentrin (A and B/kendrin), although we cannot rule out silencing of other potential pericentrin isoforms under these conditions (Flory and Davis, 2003). We typically observed silencing in 80 to 90% of treated cells (Figure 1, A–C, G, and H). Silencing of pericentrin A/B disrupted mitotic spindle organization and reduced astral microtubules, ultimately leading to the formation of monopolar spindles in most mitotic cells (Figure 1, A, A′, E, and F). The phenotype seemed to be specific for mitotic cells because interphase microtubule organization was not detectably altered (Figure 1, A and A′). γ Tubulin was reduced at spindle poles in mitotic cells, although centrioles were present; centrosomes in adjacent interphase cells retained strong γ tubulin staining (Figure 1, G and H). Selective silencing of the larger isoform of pericentrin (B) had no effect on interphase or mitotic microtubule organization (Figure 1C, C′, C″, and D), although we cannot rule out the possibility that activity of the residual protein is sufficient to support these functions. These results suggested that the phenotype observed after pericentrin A/B silencing resulted from reduction in pericentrin A, although this isoform could not be specifically targeted because it is homologous through most of its length with pericentrin B (Flory and Davis, 2003). Control cells with reduced lamin levels showed no detectable changes in any of the parameters described above (Figure 1, B, B′, E, and F; our unpublished data).

Pericentrin Interacts with the γ TuRC in Xenopus Extracts through GCP2 and 3

We next examined the relationship of pericentrin and the γ tubulin ring complex in more detail. We found that immunoprecipitation of pericentrin from *Xenopus* extracts coprecipitated several components of the γ TuRC, including γ tubulin, GCP2, and GCP3 (Figure 2A). Conversely, immunoprecipitation of γ tubulin coprecipitated pericentrin in addition to GCP2 and GCP3. Additional evidence for the pericentrin-γ TuRC interaction was obtained by showing that an HA-tagged C-terminal region of pericentrin affixed to beads could be used to specifically pull out endogenous γ tubulin and associated proteins from *Xenopus* extracts (Figure 2B, 1340–1920; our unpublished data). Moreover, the C-terminal region of pericentrin was able to disrupt the endogenous pericentrin–γ TuRC interaction when added to extracts as shown by the loss of γ tubulin from pericentrin immunoprecipitates (Figure 2C). These results demonstrate that pericentrin interacts with the γ TuRC and that the interaction is mediated by a domain at the C-terminal region of the protein.

Figure 2. Pericentrin interacts with the γ TuRC in *Xenopus* extracts. (A) Immunoprecipitation of endogenous pericentrin pulls down γ TuRC proteins (γ tubulin, GCP2, and GCP3) from *Xenopus* extracts (lane 2) and immunoprecipitation of γ tubulin pulls down pericentrin (lane 3), whereas nonspecific rabbit IgG precipitates none of these proteins (lane 1). (B) HA-tagged C-terminal domains of pericentrin bound to anti-HA beads pull down endogenous γ tubulin from *Xenopus* extracts (lanes 4 and 5), whereas beads alone and HA-tagged central and amino-terminal domains do not pull down significant γ tubulin (lanes 1–3). (C) A C-terminal domain of pericentrin (1618–1810) disrupts the interaction between endogenous pericentrin and the γ TuRC in extracts as shown by immunoprecipitation with anti-pericentrin antibodies, whereas heat-inactivated protein (h.i. 1618–1810) and phosphate-buffered saline (PBS) have no effect. Numbers in B and C represent amino acid numbers of pericentrin.
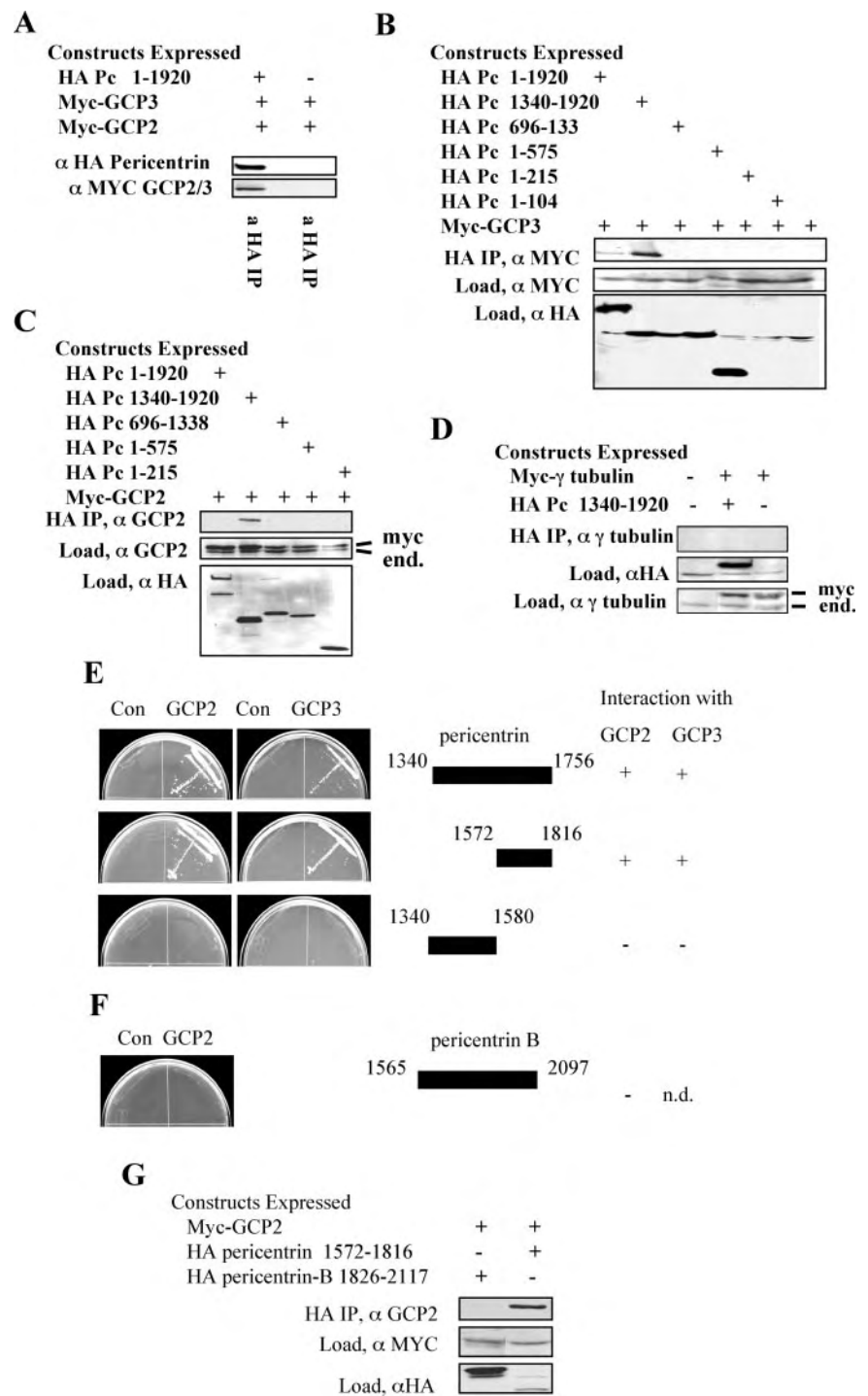
Figure 3. C-terminal domains of pericentrin interact with γ TuRC proteins GCP3 and GPC2 in vitro. (A) When coexpressed in vertebrate cells myc-tagged GCP2 and/or myc-tagged GCP3 coimmunoprecipitate with HA-tagged pericentrin (similar mobility of GCP2 and GCP3 prevents their individual identification in this experiment). (B–D) A C-terminal domain of pericentrin (amino acids 1340–1920) interacts with GCP3 (B) and GCP2 (C) but not γ tubulin (D) when coexpressed in vertebrate cells. Immunoprecipitation and immunoblotting performed as indicated. End., endogenous protein. (E) Two-hybrid analysis confirms the interaction of the pericentrin C terminus with GCP2 and GCP3 but not with γ tubulin (data not shown). (F and G) Segments of pericentrin-B corresponding to the C terminal region of pericentrin do not interact with GCP2 in two-hybrid (F) or coimmunoprecipitation experiments (G).

To determine the molecular basis of the interaction of pericentrin with the γ TuRC, we tested whether pericentrin could bind individual proteins of the complex in vitro. We found that HA-tagged full-length pericentrin and the C-terminal third of pericentrin coimmunoprecipitated myc-tagged GCP3 when the proteins were cooverexpressed in COS-7 cells (Figure 3, A and B). In parallel assays, the pericentrin C terminus coimmunoprecipitated myc-tagged GCP2 (Figure 3, A and C) but not myc-tagged γ tubulin (Figure 3D). GCP2/3 binding was specific for the C terminus of pericentrin because several domains comprising the amino terminal two-thirds of the molecule showed no interaction (Figure 3, B and C). Direct two-hybrid analysis confirmed the interaction of the pericentrin C terminus with both GCP2 and GCP3 (Figure 3E) and failed to detect an interaction with γ tubulin or amino terminal domains of pericentrin (our unpublished data). In addition, domains of the larger isoform (pericentrin B) that included the GCP2/3 interacting region of pericentrin as well as an additional exon not present in pericentrin (66% identical, 78% similarity) did not interact with GCP2 (or GCP3) by immunoprecipitation (Figure 3G) or two-hybrid analysis (Figure 3F, see

Figure 4. C-terminal fragments of pericentrin disrupt aster formation and stability and γ tubulin assembly onto centrosomes in *Xenopus* mitotic extracts. (A and B) Mitotic asters assembled in the presence of equal amounts of pericentrin 1340–1920 (B) or heat inactivated (h.i.) 1340–1920 (A). (C) Mitotic aster assembly in the presence of increasing concentrations of pericentrin (Pc) 1340–1920. (D) Quantification of aster assembly in mitotic extracts in the presence of pericentrin domains. Amount of protein added per 10 μl of extract is indicated. (1, PBS; 2, 33 ng of 1–535; 3, 12 ng of 1340–1920 ($p < 0.0001$); 4, 12 ng of h.i. 1340–1920; 5, 10 ng of peri B1826–2117; 6, 10,000 ng of BSA). Quantification of aster assembly in interphase extracts (D7–9) by using a pericentrin domain (1618–1810) that inhibits aster assembly in mitotic extracts (7. $p < 0.0001$) and is inactivated by heat (8), but has no activity in interphase extracts in the same experiment (9). (E and F) γ Tubulin assembly onto nascent centrosomes in the presence of h.i. 1340–1920 (E) or 1340–1920 (F, $p < 0.0001$). (G) Quantification of γ tubulin assembly onto centrosomes in the absence of mitotic extract (1), in extracts with 1–595 (2), 1340–1920 (3, $p < 0.0001$), 1618–1810 (4, $p < 0.0001$), or heat inactivated 1618–1810 (5). For C, D, and G, 200 sperm nuclei were counted per bar or point. (H–K) Rapid disassembly of preassembled mitotic asters over time after addition of 1340–1920. (L) h.i. Pc 1340–1920 has no effect on preassembled asters. (M and N) Ran-mediated aster assembly in extracts in the presence of h.i 1618–1810 (M) or 1618–1810 (N). A–D, H–N, microtubules or γ tubulin, red; nuclei, green. Bar (A), 10 μm for A and B; in L, 10 μm for H–N.

accession numbers gi458668 and gi31296687 for more details on sequence differences). Data from these two independent assays demonstrate that pericentrin interacts specifically with at least two members of the γ TuRC, GCP2 and GCP3. The C terminus of pericentrin seemed to bind GCP2 and GCP3 more efficiently than the full-length molecule. Similar binding patterns have been observed for other pericentrin-interacting proteins such as the dynein light intermediate chain, and they could result from increased accessibility to epitopes that are masked in the full-length protein (Tynan *et al.*, 2000).

The C Terminus of Pericentrin Disassembles Mitotic Asters and Centrosomal γ Tubulin in Xenopus Extracts

Microtubule aster formation on nascent centrosomes of sperm nuclei in *Xenopus* extracts is dependent on the recruitment of soluble γ TuRCs to these sites (Felix *et al.*, 1994; Stearns and Kirschner, 1994). Previous studies implicated pericentrin in this process (Dictenberg *et al.*, 1998; Doxsey *et al.*, 1994). To address this issue directly, we examined the effect of the GCP2/3 interacting domain of pericentrin on microtubule aster assembly in mitotic *Xenopus* extracts. Addition of this domain to extracts before the aster assembly reaction significantly reduced aster formation (Figure 4, A and B). Even after extended periods (30 min), few asters were detected, and they had few microtubules and were highly disorganized, a phenotype almost never observed in controls. Half maximal aster inhibitory activity was seen at a protein concentration ~4:1 with endogenous pericentrin (Figure 4C). No change in aster assembly was observed in the presence of the pericentrin N terminus, heat-denatured C terminus, bovine serum albumin, or buffer alone (Figure 4D, 1–6). The activity seemed to be specific for mitotic extracts as there was no detectable effect on aster assembly in interphase extracts (Figure 4D, 7–9).

The mechanism of aster inhibition was examined in more detail by monitoring recruitment of γ tubulin onto nascent centrosomes in *Xenopus* mitotic extracts as described previously (Doxsey *et al.*, 1994; Felix *et al.*, 1994; Stearns and Kirschner, 1994). The pericentrin C-terminal domain and subdomains of this protein specifically inhibited recruitment of γ tubulin onto centrosomes to the same extent and at the same concentration that prevented microtubule aster assembly and disrupted the interaction between pericentrin and the γ TuRC (Figure 4, E–G). These results suggested that the pericentrin C terminus inhibited microtubule aster formation in mitotic extracts by preventing recruitment of γ tubulin to pericentrin sites on the nascent centrosome.

To more directly test whether pericentrin anchored γ TuRCs to nascent centrosomes, we examined the effect of the pericentrin C-terminal polypeptide on asters preassembled in extracts. Within 60 s after addition of the protein, the focus of microtubules in preassembled asters was disrupted, and free microtubules were observed in the region surrounding the aster (Figure 4, H–K). By 2 min after addition of the protein, most microtubules seemed to have lost their attachment to the centrosome; the remaining microtubules were of normal length and often formed bundles. By 3–5 min, no microtubules were detected at most centrosomes. In contrast, preassembled asters exposed to heat-inactivated pericentrin C terminus (Figure 4L), other pericentrin domains, the pericentrin B homology domain, or buffer alone showed no detectable loss of centrosomal microtubules, no change in microtubule organization, and few to no free microtubules in the vicinity of the aster. Pericentrin C-terminal peptide was just as effective at disrupting preexisting asters as it was at inhibiting their assembly, through a range

of test concentrations. Pericentrin C terminal peptide caused loss of γ tubulin from preassembled centrosomes within the same time frame that it caused loss of microtubules from asters (90% reduction in 5 min). These results indicate that the pericentrin C terminus disrupts the interaction of γ TuRCs with centrosomes releasing the complexes and attached microtubules.

Microtubule asters can form in *Xenopus* extracts by a centrosome-independent pathway that requires the Ran GTPase (reviewed in Dasso, 2002). Ran-mediated aster assembly can be inhibited by a dominant negative form of Ran and enhanced by a dominant active form of the protein. Under conditions that resulted in rapid disassembly of mitotic asters, the pericentrin C terminus did not significantly affect Ran-mediated aster assembly even after extended periods of incubation (Figure 4, M and N). Thus, although formation of Ran asters requires γ tubulin (Wilde and Zheng, 1999), it seems to be less dependent on pericentrin than does centrosome-mediated aster assembly.

Mapping the GCP2/3 Binding Domain and Aster-disrupting Activity of Pericentrin

We further defined the pericentrin–GCP2/3 interaction site and made point mutants that inhibited the pericentrin–GCP2/3 interaction. Using directed two-hybrid and coimmunoprecipitation analyses, we identified a subdomain of the C terminus that was required for strong GCP2/3 binding in both assays (Figure 5A, consensus). We identified a point mutation in this domain with significantly reduced binding to GCP2 in vitro and lacked aster activity in *Xenopus* extracts. GCP2 and GCP3 bind cooperatively to pericentrin with in the consensus region because myc-tagged GCP2 showed cooperative binding to HA-tagged pericentrin in the presence of GCP3 (Figure 5C). In functional assays, pericentrin domains that bound GCP2/3 showed aster inhibitory activity in *Xenopus* extracts (Figure 5A). Those that did not interact with GCP2/3 lacked aster inhibitory activity, including the pericentrin mutant, a domain of pericentrin B containing all pericentrin sequences required for activity (Figure 5A), and several pericentrin domains outside the GCP2/3 interacting domain (Figure 5A, consensus). The strong correlation between regions of pericentrin that interacted with GCP2/3 and those that showed mitotic aster and γ TuRC-disrupting activity indicated that pericentrin was required for anchoring γ TuRCs to centrosomes in *Xenopus* mitotic extracts.

To further address differences in GCP2/3 binding between pericentrin (A) and pericentrin B, we excised most of an extra exon (and some additional sequences) that is present in the homologous region of pericentrin B. Truncated pericentrin B proteins lacking the amino acids encoded by these sequences had weak GCP2 binding activity (Figure 5, A and D), suggesting that pericentrin B binding to the γ TuRC in this region may be blocked by incorporation of an extra exon.

GCP2/3 Interacting Domains of Pericentrin Disrupt Mitotic Asters and Spindles in Vertebrate Cells

We next tested whether the GCP2/3-interacting domains of pericentrin affected microtubule organization in vertebrate cells. We found that these domains had no detectable effect on the organization or nucleation of microtubules or the organization of centrosomes in interphase SAOS cells (Figure 6A). However, the same domains disrupted microtubule structures in mitotic cells (Figure 6, B–K). The most common phenotype was monopolar spindles, which represented ~15% of all mitotic cells at early times posttransfection
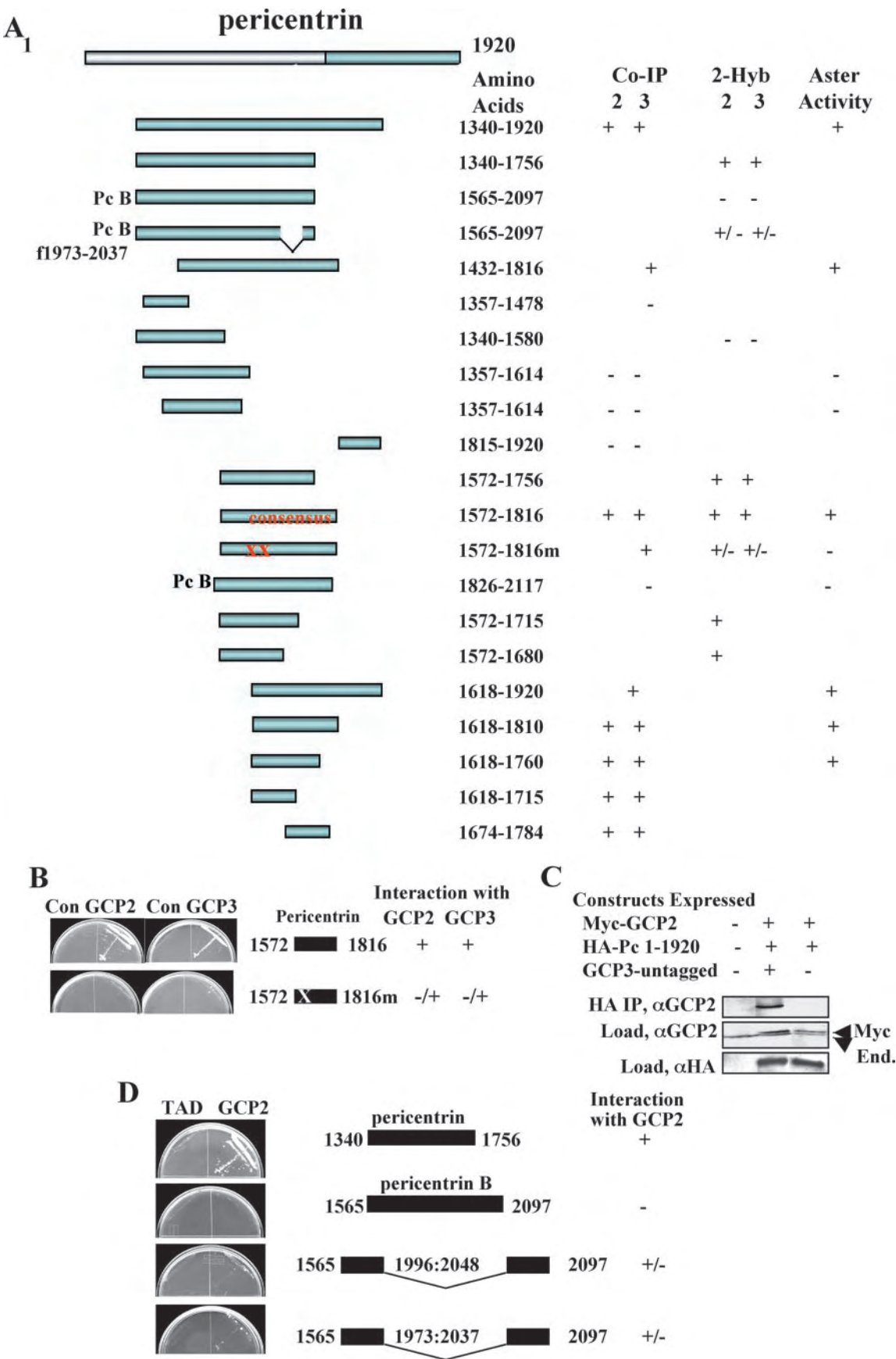
Figure 5.

(20–22 h) and increased to ~90% at later times (44 h post-transfection, Figure 6, H, H', and M). Most monopolar spindles had two duplicated and separated centrosomes. We also observed spindles with reduced numbers of centrosome-associated astral microtubules (Figure 6, C', G, J, and K'), bipolar spindles with shortened pole-to-pole axes (Figure 6, C' and G, minispindles) and half spindles with single focused poles (Figure 6, I–I''). In many spindles, we observed a decrease in the centrosome level of γ tubulin (Figure 6, E and I) and other centrosome proteins (Figure 6C), although the proteins were never reduced to undetectable levels. Pericentrin domains that bound both GCP2 and GCP3 induced the same defects, and those that did not interact had little or no effect (Figure 6, K–K''). Moreover, we were unable to detect aster inhibitory activity in *Xenopus* extracts (Figures 4D and 5A), disruption of spindle organization (Figure 6, M–M') or apoptosis (below) associated with the homologous region of pericentrin B, suggesting that these two molecules may not be functionally analogous. Together, these results suggest that uncoupling of the pericentrin A–γ tubulin interaction in mitotic cells caused a reduction in the centrosome-associated γ TuRCs and disrupted astral microtubules and spindle organization, ultimately producing monopolar spindles.

Overexpression of the GCP2/3 Binding Domain of Pericentrin and Reduction in Pericentrin Levels Both Induce G2/Antephase Delay and Apoptosis

During the course of these studies, we observed a marked reduction in cell density in cultures transfected with the GCP2/3 binding domain (Figure 7, A and A'). Typically, half the cells detached from their substrate by 44 h, whereas there was little change in cell density before protein expression (Figure 7A', 20 h). To investigate this further, we examined cells for apoptosis and found that a significant fraction of the cells stained with an apoptosis-specific marker that detects a caspase 3 product of cytokeratin 18 produced early in apoptosis (Figure 7A'', M30); control cells showed low levels of M30 staining.

Apoptosis required that cells be actively cycling, because we did not detect apoptosis when cells were plated at high density to induce G_1/G_0 arrest during the period of protein expression. In cycling cells of several different origins, we observed a low mitotic index (Figure 7C) suggesting that cells were delayed at some point in the cell cycle. We found that cells accumulated in a premitotic stage based on their ability to stain for a form of histone H3 that is phosphorylated by aurora B in early mitotic cells (Swedlow and Hirano, 2003; Hans and Dimitrov, 2001); control cell staining was significantly lower (Figure 7, D and F). The cell cycle period between late G2 and mitosis (before chromosome condensation occurs) is termed antephase (Pines and Rieder, 2001). Antephase arrest was linked to apoptosis because most early mitotic cells (phospho-H3-positive) were also early apoptotic (Figure 7G, M30-positive). Moreover, most centrosomes in apoptotic cells seemed duplicated and separated (Figure 7H, two γ tubulin spots), consistent with cells in late G2 or early prophase.

To confirm the link between cell cycle arrest and cell death, we microinjected cDNA into nuclei of COS cells arrested in S phase by thymidine block. Approximately 8 h after release from the block cells entered mitosis. At this time, a significant proportion of cells expressing the GCP2/3 binding domain of pericentrin expressed the M30 antigen or detached from the substrate whereas control cells remained attached and often increased in number (Figure 8, A, B, and D). Cell loss was cell cycle specific because premitotic cycling cells or cells kept under S phase arrest remained viable and adherent (Figure 8C). These results suggested that uncoupling the pericentrin–γ TuRC interaction and disruption of astral microtubules induced apoptosis at the G2/M transition. (Figure 8C).

We reasoned that if apoptosis resulted from a cellular defect common to both overexpression and reduction of pericentrin, we should observe cell cycle arrest and apoptosis after pericentrin silencing. Significant cell death was in several cell types knocked down for pericentrin A and B at 48–72 h posttreatment (our unpublished data). Pericentrin A/B silencing also induced a significant increase in antephase, and a decrease in mitotic index 48–72 h after protein silencing (Figure 7, C and D). These provide further support for the idea that antephase arrest and apoptosis may be caused by disruption of the pericentrin–γ tubulin interaction.

DISCUSSION

Our previous results demonstrated that pericentrin and γ tubulin interacted in *Xenopus* extracts and that the proteins were in proximity at centrosomes in vertebrate cells, suggesting that they interacted at this site as well (Dictenberg *et al.*, 1998). The additional data provided in this study show that pericentrin interacts with the γ TuRC via domains that bind GCP2 and GCP3 and that this interaction is important for microtubule organization in mitotic cells. The results of this study are consistent with our previous work showing that pericentrin overexpression induces severe spindle defects (Purohit *et al.*, 1999). We propose a model in which pericentrin acts as a scaffold for anchoring γ TuRCs at mitotic centrosomes/spindle poles. This interaction seems to be required not only for astral microtubule organization but also for maintaining spindle bipolarity and for mitotic entry. The monopolar spindles and "minispindles" induced by disruption of the pericentrin-GCP2/3 interaction, indicate that pericentrin anchoring of γ TuRCs also may play a role in organizing microtubules of the central spindle.

Figure 5 (facing page). Summary of GCP2/3 binding and aster inhibitory activity of pericentrin domains. (A) Binding and aster activity of various pericentrin constructs. CoIP, coimmunoprecipitation; 2-hyb, yeast two-hybrid; aster, aster inhibitory activity; consensus, smallest domain identified in both coIP and 2-hyb that has high-affinity binding activity to both GCP2 and GCP3; XX, E to A mutations in 1613 and 1615, Pc B ϕ 1973–2037 lacks an exon encoding the indicated amino acids. Although variable, interactions of all affinities were scored as + unless they were at the limit of detection (then scored as +/−). No markings such as + or − denote data not acquired for these parameters. Proteins were considered positive in the aster inhibition assay if they showed at least 31% reduction in aster assembly relative to control activity. For clarity, the pericentrin B constructs are arbitrarily sized and aligned with homologous regions of pericentrin. (B) Yeast two-hybrid data showing significantly reduced binding of mutant pericentrin domain for GCP2 and GCP3. (C) Cooverexpression, coimmunoprecipitation data showing enhanced binding of GCP2 to pericentrin 1–1920 in the presence of GCP3 (see Figure 2 legend for details). Con, control. (D) Yeast two-hybrid data showing binding of GCP2 by a pericentrin A fragment and lack of GCP2 binding by the homologous pericentrin B fragment as well as mutants lacking a pericentrin B specific exon (see MATERIALS AND METHODS for details).
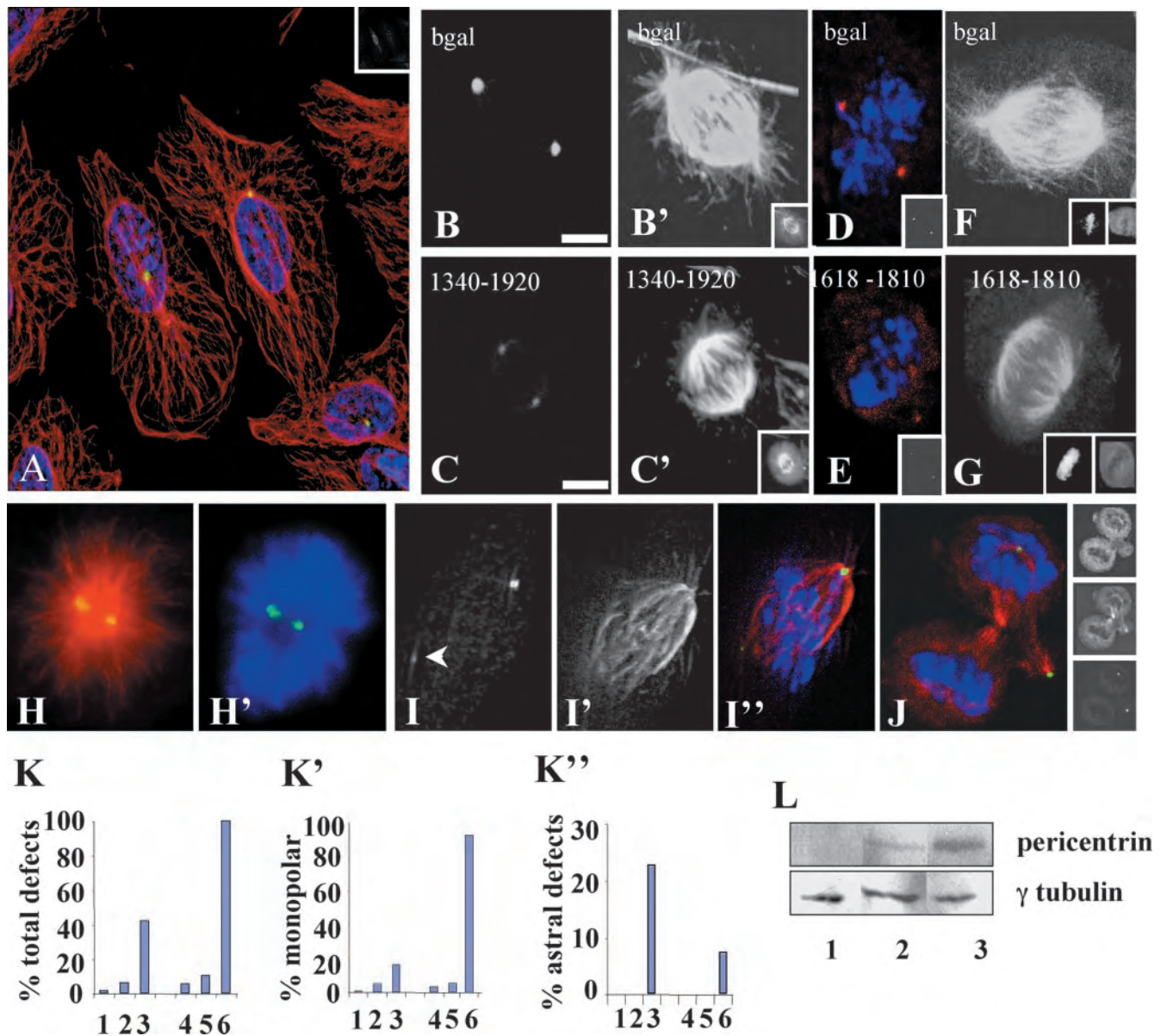
Figure 6. GCP2/3 binding domain of pericentrin affects astral microtubules and spindle organization in vertebrate cells. (A) Interphase cell expressing pericentrin 1680–1810 (inset, top right) shows no difference in microtubule organization compared with surrounding control cells (red, microtubules; blue, DNA stained with DAPI; yellow, 5051 centrosome staining). (B and B') Control mitotic cell expressing β-galacto-sidase. (C and C') Cell expressing pericentrin 1340–1920 and spindles with reduced astrals and pole-to-pole distance (C', compare with B and B'). Insets at bottom right of B', C' show protein expression. (D) β-Galactosidase–expressing control cell. (E) Cell expressing pericentrin 1680–1810 shows reduced γ tubulin at spindle poles (compare with D). D and E, γ tubulin (red), DNA (blue), insets show 5051 staining. (F) β-Galactosidase–expressing cell. (G) Cell expressing 1680–1810 (inset, bottom right) shows reduced astral microtubules and decreased pole-to-pole distance compared with F. DNA, insets, left. Overexpressed protein, insets, right. (H) Monopolar spindle in cell expressing 1680–1810 showing centrosomes (yellow, 5051) and microtubules (H) or DNA (H'). (I) Spindle from cell expressing 1680–1810 with one tiny spindle pole (arrowhead, 5051) and unfocused microtubules at this pole (I', merge, I''). (J) Telophase cell expressing 1680–1810 undergoing tripolar division. One nascent daughter cell lacks a centrosome (bottom). Images at right show protein expression (top), microtubules (middle) and centrosomes (bottom). (A–J) Immunofluorescence images of SAOS cells. All images except H and H' are shown with deconvolution. Paired images were stained in parallel and collected on the same day, without modification to the laser or acquisition settings between images. (K–K'') Graphs showing percentage of transfected mitotic SAOS cells with total mitotic defects (K), monopolar spindles (K'), and reduced or absent astral microtubules (K'') 1–3, 20 h posttransfection; 4–6, 40 h posttransfection. 1, nontransfected mitotics, n = 368; 2, βgal, n = 82; 3, 1618–1810, n = 31; 4, Peri B 1826–2117; n = 95. 5, βgal, n = 39. 6; 1618–1810, n = 14. p values comparing βgal- and 1618–1810–expressing cells were calculated using the Student's *t* test for both time points. K, $p < 0.0001$ at 22 h; $p < 0.0001$ at 44 h. K', $p = 0.049$ at 22 h; $p < 0.0001$ at 44 h. (L) Soluble pericentrin is more abundant in mitotic cells. Western blot of HeLa whole cell lysates from asynchronous cells (lane 1), from cells treated 4 h with nocodazole (lane 2), and from mitotic cells after shake-off (lane 3). Total protein loads were normalized for each lane using the Bio-Rad protein assay.

Centrosomal Anchoring of γ TuRCs by Pericentrin Is Required for Mitotic Microtubule Aster Organization in Xenopus Extracts and Somatic Cells

Our results indicate that pericentrin anchoring of γ TuRCs at centrosomes is required for mitotic aster organization. If anchoring is disrupted, γ tubulin is dramatically depleted at mitotic centrosomes in *Xenopus* extracts and reduced at spindle poles in somatic cells. The more dramatic loss of centrosomal γ tubulin from *Xenopus* asters suggests that pericentrin plays a more dominant role in the organization of γ TuRCs at centrosomes in this system and perhaps in embryonic systems in general. We have not investigated the fate of γ TuRCs once dissociated from centrosomes, although one possibility is that they remain attached to the minus ends of microtubules where they could cap microtubule growth (Wiese and Zheng, 2000). In somatic cells, a fraction of γ tubulin remains at centrosomes/spindle poles under conditions that disrupt the GCP2/3-pericentrin interaction. This fraction could be anchored by other proteins that have been shown to bind γ TuRC components such as AKAP450, pericentrin B (Takahashi *et al.*, 2002) Nlp (Casenghi *et al.*, 2003), and centrosomin (Terada *et al.*, 2003)

In this study, we map the GCP2/3 binding site of pericentrin to the C terminus of the protein, a region that shows no apparent homology to AKAP450, Spc110, Spc72, or CP309 (Kawaguchi and Zheng, 2003; Takahashi *et al.*, 2002), although it is conserved between mouse, human, and rat (66–75% identical, 78–84% similarity). Whereas the amino terminus of pericentrin B binds GCP2 (Takahashi *et al.*, 2002) (W. Zimmerman and S. Doxsey, unpublished observations), a similar region in the smaller pericentrin isoform does not, perhaps because it lacks exons found in pericentrin B. More information on the GCP2/3 interacting domain will require mapping these sites in all the GCP2 binding proteins.

The phenotype observed with the GCP2/3-pericentrin disrupting polypeptides and after pericentrin silencing is similar in many respects to that seen after functional abrogation of γ tubulin and other proteins of the γ TuRC. Under these conditions, centrosomes in *Caenorhabditis elegans* and *Drosophila* embryos were compromised in their ability to form mitotic asters (Hannak *et al.*, 2002; Strome *et al.*, 2001), separate from one another (Barbosa *et al.*, 2003; Sampaio *et al.*, 2001), and organize meiotic and mitotic spindles (Sunkel *et al.*, 1995; Barbosa *et al.*, 2000, 2003). It is of interest that mitotic asters in some of these systems formed in the absence of γ tubulin or other γ tubulin ring complex proteins (Strome *et al.*, 2001; Hannak *et al.*, 2002; Barbosa *et al.*, 2003). This is in contrast to our results in *Xenopus* extracts where microtubule asters did not form in the presence of the pericentrin interacting domain of GCP2/3 even after extended periods (30 min). Moreover, preformed mitotic asters were rapidly disassembled after addition of this polypeptide. Future studies will be required to determine whether pericentrin and γ tubulin are more critical for mitotic aster formation in *Xenopus* extracts than in the other systems, or whether uncoupling γ tubulin from pericentrin prevents both γ tubulin-mediated microtubule nucleation and nucleation by a proposed γ tubulin-independent pathway (Hannak *et al.*, 2002).

Pericentrin Is Not Essential for Assembly and Anchoring of γ TuRCs at Interphase Centrosomes

The GCP2/3-interacting pericentrin domains described in this study had no detectable effect on assembly of asters in interphase extracts prepared from *Xenopus* or in interphase somatic cells. Moreover, silencing of both isoforms also had no apparent effect on localization of γ tubulin at the centrosome or microtubule organization in interphase cells. This suggests that the protein does not play a major role in γ tubulin assembly or anchoring at interphase centrosomes but rather that the aster-organizing function of pericentrin is mitosis specific. Because both proteins are normally present at the centrosome throughout the cell cycle, we cannot conclude that they do not interact during interphase. Only that this specific interaction is not necessary for γ tubulin localization. It has been shown that γ tubulin and associated proteins are crucial for microtubule nucleation from interphase centrosomes (Joshi *et al.*, 1992; Hannak *et al.*, 2002). It is thus likely that proteins other than pericentrin provide microtubule-anchoring sites at centrosomes in interphase cells.

Other Proteins Involved in Centrosomal γ TuRC Anchoring and Microtubule Organization

Several other proteins play a role in centrosome organization and microtubule nucleation. However, their ability to directly anchor components of the γ TuRC and thus serve as molecular scaffolds for tethering these complexes to centrosomes has not been demonstrated. These include the centrosome proteins Asp (do Carmo Avides and Glover, 1999), NuMA (Merdes *et al.*, 1996), TPX-2 (Wittmann *et al.*, 2000; Garrett *et al.*, 2002), SPD-5 (Hamill *et al.*, 2002), PCM-1 (Dammermann and Merdes, 2002), Sas-4 (Kirkham *et al.*, 2003) centrosomin (Megraw *et al.*, 1999; Terada *et al.*, 2003), and several regulatory molecules, including Aurora A (Hannak *et al.*, 2001; Giet *et al.*, 2002), Polo (Lane and Nigg, 1996; Barbosa *et al.*, 2000), PP1 (Katayama *et al.*, 2001), and PP4 (Sumiyoshi *et al.*, 2002).

Some of these proteins play a critical role in a centrosome-independent spindle assembly pathway mediated by the Ran GTPase (see Dasso, 2002) including NuMA (Nachury *et al.*, 2001; Wiese *et al.*, 2001) and TPX-2 (Gruss *et al.*, 2001). This is in contrast with pericentrin, which seems to be critical for assembly of mitotic asters but not Ran-mediated asters. In this regard, the proposed function of pericentrin in aster formation also differs from that of epsilon tubulin, which seems to be required for centrosome-independent but not centrosome-dependent microtubule aster formation (Chang *et al.*, 2003). From this discussion, it seems that different molecules are required to organize asters in centrosome-dependent and -independent pathways as well as at different stages of the cell cycle.

Regulation of the Pericentrin–GCP2/3 Interaction

Pericentrin, γ tubulin, and γ tubulin-associated proteins are localized to centrosomes throughout the cell cycle (Stearns *et al.*, 1991; Zheng *et al.*, 1991; Dictenberg *et al.*, 1998). However, the pericentrin–GCP2/3 interaction seems to be involved in γ TuRC anchoring only during mitosis. This suggests that the interaction of pericentrin and γ TuRCs is regulated. The mechanism and regulation of cell cycle-specific binding between these centrosome components is unknown. One model is that γ TuRCs are anchored to different centrosome scaffold proteins at different cell cycle stages and that these interactions are regulated in a cell cycle-dependent manner. For example, the γ TuRC binding activity of pericentrin could be regulated by phosphorylation by mitotic kinases. γ TuRC binding also could be regulated at least in part, through differential patterns of protein expression. Consistent with this idea is the observation that pericentrin, which is expressed primarily in mitosis and in tissues that are highly proliferative (Doxsey *et al.*, 1994; Figure 6N), has a mitotic phenotype. Future experiments will be required to determine the contribution of these and other centrosome
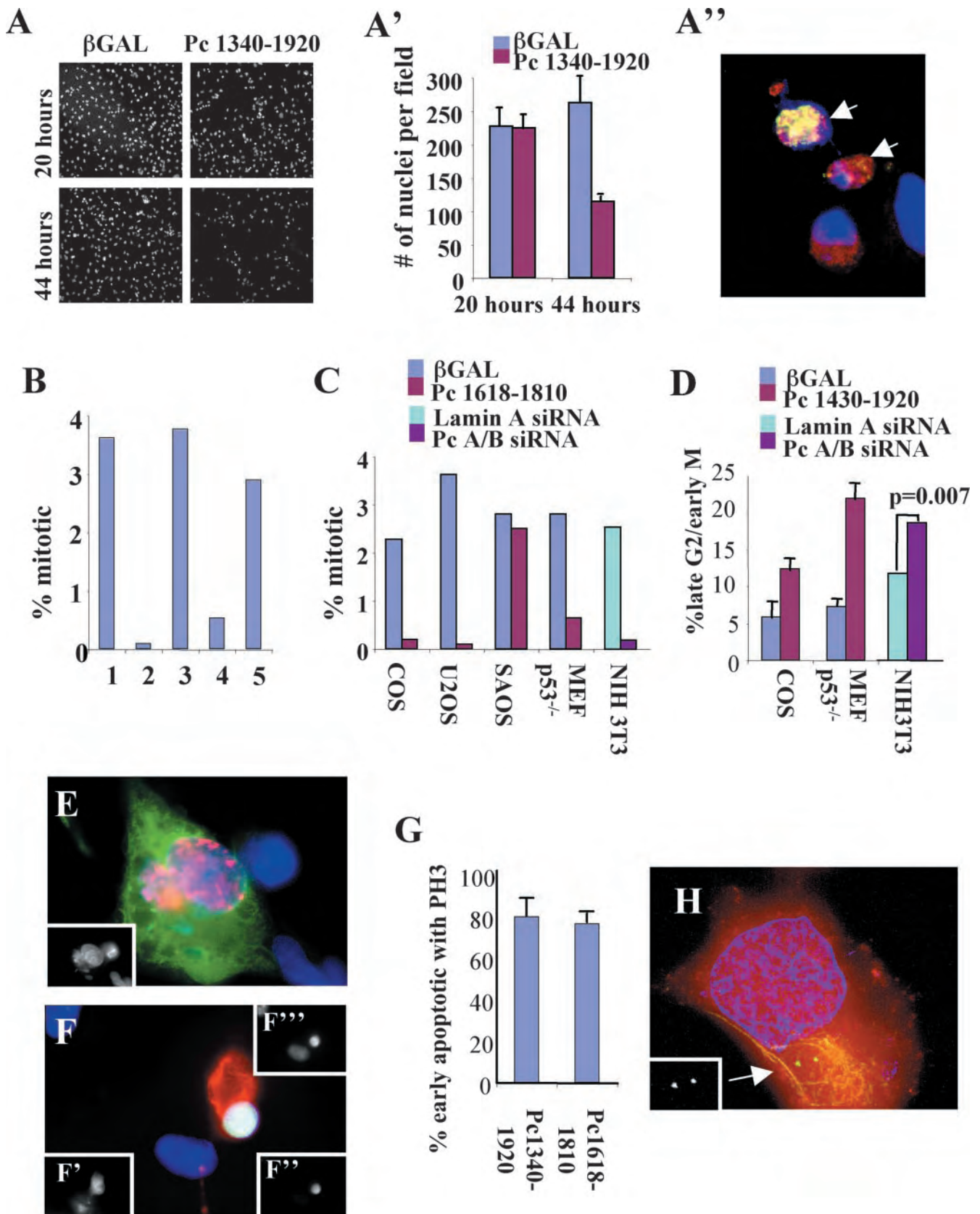
Figure 7. Overexpression of GCP2/3 binding domain or silencing of Pc A/B induces cell cycle arrest and apoptosis at the G2/M phase of the cell cycle. (A) Cells expressing the GCP2/3 binding domain are lost through apoptosis. Low-magnification image of COS cells stained with DAPI showing cell loss when 1340–1920 is expressed for 44 h compared with nonexpressing cells (20 h) or β-galactosidase (βgal)–expressing cells. (A′)

proteins in the anchoring of γ TuRCs to centrosomes at different cell cycle stages.

G2/Antephase Delay and Apoptosis

G2 accumulation of cells expressing the GCP2/3 binding domain of pericentrin or after silencing of pericentrin A/B suggests that disruption of the pericentrin γ TuRC interaction in vivo elicits a checkpoint response at this time in the cell cycle. Recent studies have implicated γ tubulin as well as the Spc110p homologue Pcp1p in regulation of the metaphase to anaphase transition (Prigozhina *et al.*, 2004; Rajagopalan *et al.*, 2004), but this is the first study suggesting a role for these or related molecules in regulation of mitotic entry. We do not yet know what this checkpoint may be monitoring. We favor a model in which the checkpoint senses spindle pole assembly/centrosome maturation because disruption of the γ tubulin–pericentrin interaction disrupts spindle pole assembly and possibly centrosome maturation, which increases in size fourfold between G2 and early prophase (Piehl *et al.*, 2004), concurrent with the onset of γ tubulin mislocalization and antephase arrest that we observe.

Our results showing that pericentrin A/B silencing has no significant affect on interphase microtubule arrays confirms previous work (Dammermann and Merdes, 2002). In this earlier study, the authors did not address mitotic defects most likely because a G2 checkpoint is activated, apoptosis follows, and mitotic cells are rarely observed, a phenomenon that we have encountered in two of the cell lines used by these authors; U2OS and HeLa (Figure 7C; data not shown). In this study, we overcame this problem by using a cell line that that apparently lacks this checkpoint and fails to undergo apoptosis.

Apoptosis is commonly observed after checkpoint activation if a cellular imbalance cannot be repaired. We have not determined which molecular pathway is involved in the G2/antephase arrest identified in this study. DNA damage induces two molecularly distinct pathways involved in G2 arrest, one ATM dependent, the other ATM independent (Xu *et al.*, 2002). Cellular insults other than DNA damage
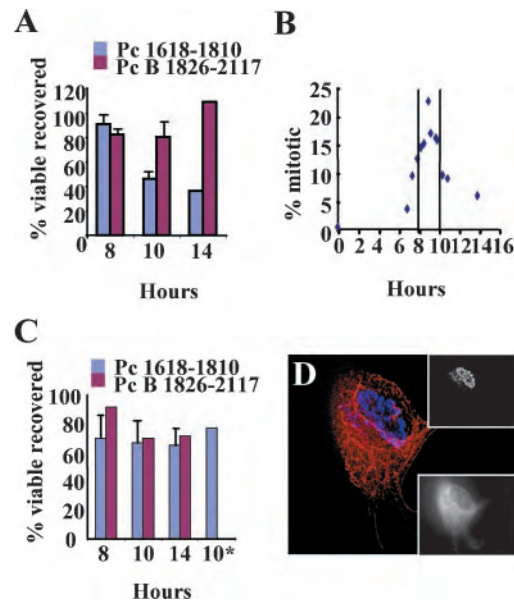
Figure 8. COS cells expressing Pc1618–1810 undergo apoptosis during the G2/M transition. (A) Cells expressing 1618–1810 undergo apoptosis at mitosis. Microinjected cells released from a double thymidine block were stained for DNA (DAPI) and overexpressed protein (250 cells/bar). 1618–1810–expressing cells were apoptotic or detached 8–10 h after injection, whereas control cells (Pc B1826–2117) increased in number. At 14 h, p value comparing the two treatments, p < 0.0001 (B) Mitotic index of COS cells after release from double thymidine block as in A (peak, 9 h). (C) 1618–1810–expressing cells arrested in S phase do not undergo apoptosis. Microinjected cells retained in thymidine for the times indicated. No loss of cells was observed 8–14 h later. 10*, microinjected cells arrested in S phase for 6 h and then released from the block for 4 h (10 h total, p = 0.25). (D) Immunofluorescence image of an apoptotic cell expressing 1618–1810 at 10 h postinjection as in A. D (overlay), DNA (blue and upper inset), M30 (red), overexpressed protein (lower inset).

Figure 7 (cont). Quantification of cells in A (mean and SD, 10 fields). (A″) Image of COS cells from A stained for DNA (blue), 1340–1920 (red) and M30 cytodeath (green). Arrows, cells with both 1340–1920 and M30. (B) Mitotic index of U2OS cells expressing: 1, βgal; 2, 1618–1810 p < 0.001; 3, Pc B1826–2117 p = 0.437; 5, 1572–1816 p = 0.011; 6, 1572–1816m p = 0.226 (n = 1000 cells/bar at 40–44 h posttransfection). p values were calculated using the *t*-test relative to βgal controls. (C) Mitotic index in indicated cell types overexpression the indicated constructs or treated with siRNA. p values calculated as described above: COS, p < 0.001; U2OS, p < 0.001; SAOS, p = 0.479; Mefp53−/−, p = 0.001; NIH3T3 (siRNA), p = 0.0004. (D) Cells expressing GCP2/3 binding domain constructs or treated with Pc A/B siRNA have a greater proportion of late G2 cells. Shown are mean and SD of three experiments or p value based upon scoring from 1000 treated cells. Cells immunostained for overexpressed protein (green), phosphorylate histone H3 (red), and DAPI (blue). (E) Antephase cell overexpressing 1340–1920 (stains for phosphorylated histone H3 and does not show condensed chromatin). Inset, DAPI. (F) Apoptotic antephase cells expressing 1340–1920. M30 (red), phosphorylated histone H3 (green; inset, bottom right), DNA (blue; inset, above), overexpressed protein (inset, bottom left). (G) Graph showing that most early apoptotic cells expressing GCP2/3 binding domains stain for phosphorylated histone H3. Shown are mean, SD, n = 3 experiments. (H) Early apoptotic cell expressing 1340–1920 stained for centrosomes (5051, green), DNA (blue), HA pericentrin (red), M30, yellow. Inset 5051. Imaged as in Figure 5.

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also can induce late G2 arrest, including microtubule disruption, hypothermia, fluoride treatment, and viral protein expression (Pines and Rieder, 2001; Tyler *et al.*, 2001; Elder *et al.*, 2002; Mikhailov and Rieder, 2002).

Antephase delay and subsequent apoptosis also can be activated through pathways that include p53 and Rb. Our data demonstrate that that p53 is not involved because primary MEFs lacking p53 retain the checkpoint response. SAOS cells, which do not arrest and apoptose have been reported to lack Rb (Scolnick and Halazonetis, 2000). We are currently testing the role of Rb in the checkpoint response. We also are investigating other mechanisms for inducing apoptosis. For example, apoptosis can be triggered by mislocalization of antiapoptotic signals from centrosomes (Li, 1998). γ Tubulin has recently been shown to associate with DAP-like kinase, which is implicated in apoptosis (Preuss *et al.*, 2003). However, the role of γ tubulin mislocalization from centrosomes and induction of apoptosis through DAP-like kinase has not been explored. Mislocalization of survivin and other antiapoptotic proteins from centrosomes can induce apoptosis (Reed and Reed, 1999; Piekorz *et al.*, 2002; Sandal *et al.*, 2003). Additional studies will be required to identify the proteins and pathways involved in the apoptotic response observed after disruption of the pericentrin–GCP2/3 interaction.

ACKNOWLEDGMENTS

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